Influence of the hepatitis C genotype on the antiviral immune response

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1 Introduction

1.1 Hepatitis C virus (HCV) infection

HCV was first isolated in 1989 (Choo et al., 1989) after its existence had first been postulated 15 years earlier to explain the occurrence of transfusion-associated liver disease in individuals infected with neither the hepatitis A or B viruses (Alter et al., 1975; Feinstone et al., 1975; Prince et al., 1974). According to the World Health Organization (WHO) approximately 130-150 million people worldwide are currently infected with HCV and 3-4 million new infections occur every year causing both acute and chronic liver infection (WHO, 2014). Acute infection remains asymptomatic in the vast majority of cases and 15-45% of patients are able to spontaneously clear the infection within 6 months. The 55-85% of patients who develop chronic infection have a great risk of developing liver fibrosis, cirrhosis or hepatocellular carcinoma (HCC). Progression of liver disease occurs over several decades and 10-20% of patients develop cirrhosis over 20–30 years of infection (Esteban et al., 2008). In cirrhotic patients the risk of developing HCC is approximately 1-5% per year and patients diagnosed with HCC have a 33% probability of death during the first year after diagnosis (Afdhal, 2004; Fattovich et al., 1997; Mutimer et al., 2014; Poynard et al., 1997; Thein et al., 2008).

If symptoms occur during the acute phase of infection they are usually mild and unspecific like fatigue, myalgia, low-grade fever, nausea and vomiting, even though jaundice is also observed (WHO, 2014). Interestingly, asymptomatic infection results in the establishment of a chronic infection in 85-90% of cases, whereas spontaneous viral clearance is more frequently observed in patients with jaundice and other severe clinical signs of hepatitis (Gerlach et al., 2003). The presence of jaundice might be an indicator for a successful host immune response by cytotoxic cells that results in clearance of the virus.

1.2 HCV and its genome organisation

HCV is a member of the Flaviviridae family, which also includes yellow fever and dengue, and is the prototype virus in the *Hepacivirus* genus (Moradpour et al., 2007), which now also includes the GB-virus B and the recently identified nonprimate, rodent and bat hepaciviruses (NPHV (Kapoor et al., 2011), RHV (Kapoor et al., 2013)

and BHV (Quan et al., 2013)). It has a 9.6 kb large positive-strand ribonucleic acid (RNA) genome, which contains 5' and 3' untranslated regions (UTRs) flanking a single open reading frame (ORF) of approximately 3000 amino acids (aa) (figure 1.1) (Bartenschlager et al., 1993). The 5' UTR is a highly conserved region and contains four different domains. Domains II-IV together with the first 24-40 nucleotides of the core coding region constitute the internal ribosomal entry site (IRES), which mediates the translation of the ORF (Otto and Puglisi, 2004; Tsukiyama-Kohara et al., 1992). Translation of the viral genome gives rise to a polyprotein that is cleaved by viral and host proteases into 10 viral proteins: the structural proteins core, E1 and E2, and the non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B.

The 3' UTR located downstream of the coding region is composed of a short variable region, a poly(U/UC) tract with an average length of 80 nucleotides and an RNA element called the X-tail, which is almost invariant. The conserved elements in the 3' UTR are essential for HCV replication (Kolykhalov et al., 1996; Tanaka et al., 1996; Yanagi et al., 1999).

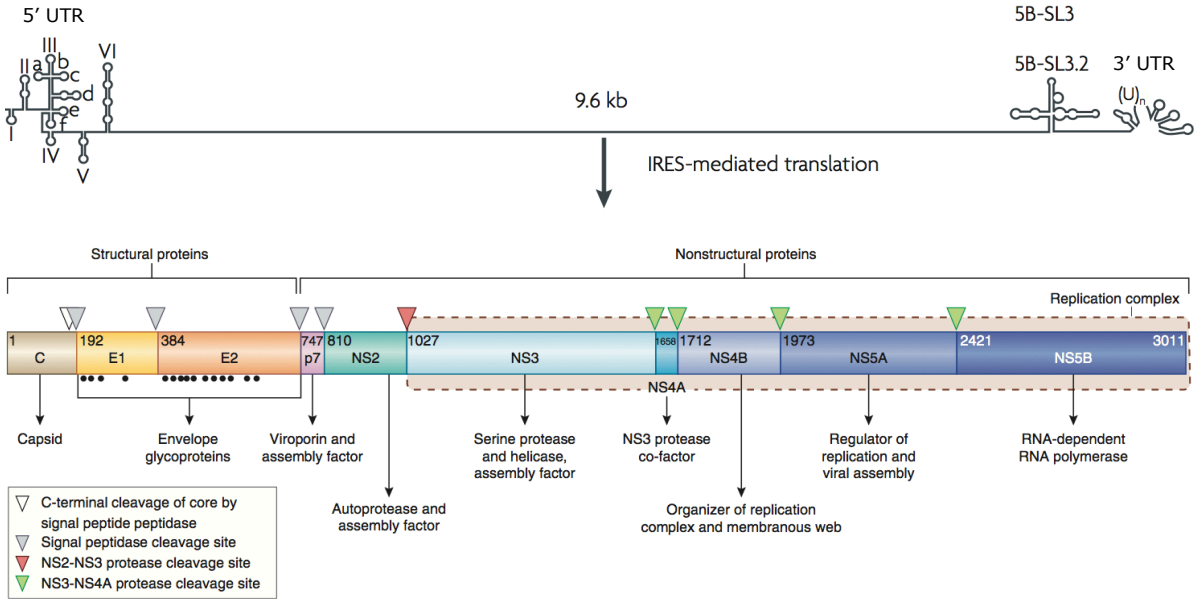

Figure 1.1: HCV genome organisation and polyprotein processing

The HCV genome contains one ORF flanked by 5' and 3' UTRs (top). IRES-mediated translation yields a polyprotein that is further co- and post-translationally cleaved by host and viral proteases as indicated (triangles). Glycosylation of the envelope proteins (black dots) and the functions of the individual HCV proteins are indicated. Modified from (Moradpour et al., 2007; Scheel and Rice, 2013).

1.3 HCV viral life cycle

1.3.1 Entry and uncoating

Entry of HCV into hepatocytes is a multistep process requiring the viral envelope glycoproteins as well as several cellular attachment and entry factors (figure 1.2 a and b).

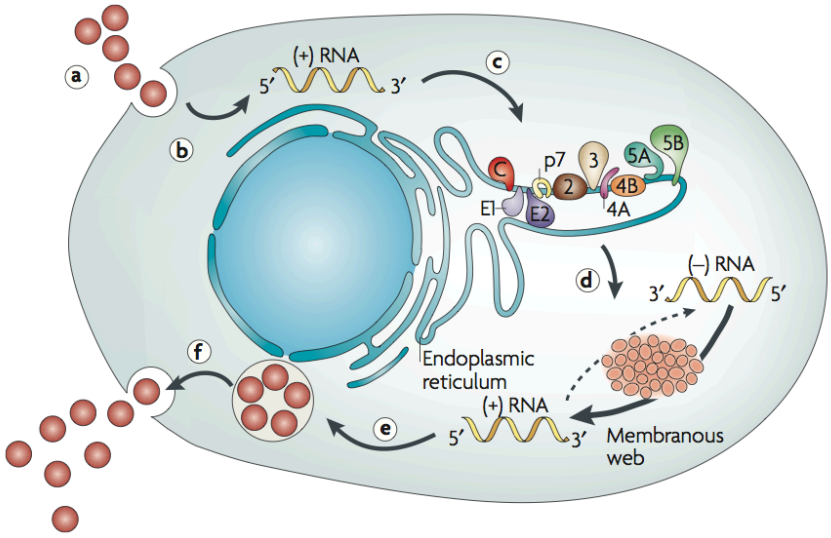

Figure 1.2: HCV viral life cycle

Virus binding and internalization (**a**); cytoplasmic release and uncoating (**b**); IRES-mediated translation and polyprotein processing (**c**); RNA replication (**d**); packaging and assembly (**e**); virion maturation and release (**f**). The topology of HCV structural and non-structural proteins at the endoplasmic reticulum (ER) membrane is shown schematically. Modified from (Moradpour et al., 2007).

Initial low affinity cell binding has been attributed to the low-density lipoprotein (LDL) receptor and glycosaminoglycans (GAG) (Agnello et al., 1999; Barth et al., 2003), followed by E1-E2 interaction with the co-receptors scavenger receptor class B type I (SR-BI) (Scarselli et al., 2002) and the tetraspanin cluster of differentiation (CD) 81 (Pileri et al., 1998). In addition, the tight-junction proteins claudin-1 (CLDN1) (Evans et al., 2007) and occludin (OCLN) (Ploss et al., 2009) as well as the receptor tyrosine kinases epidermal growth factor receptor (EGFR) and ephrin receptor type A2 (EphA2), which have both been associated with the possible modulation of the interactions between CD81 and CLDN1 (Lupberger et al., 2011), are required for entry. Virion-associated cholestrol seems to be involved at a late stage of HCV entry by interacting with the Niemann-Pick C1–like 1 (NPC1L1) cholesterol absorption

receptor (Sainz et al., 2012). Internalization occurs via clathrin-mediated endocytosis (Blanchard et al., 2006) followed by pH-dependent membrane fusion within an acidic endosomal compartment leading to the release of the HCV genome into the cytoplasm (Koutsoudakis et al., 2006; Tscherne et al., 2006). Fusion is probably mediated by E2, which is a predicted class II fusion protein (Krey et al., 2010) and has been shown to be primed for activation at acidic pH levels (Sharma et al., 2011).

1.3.2 Translation and polyprotein processing

Translation in association with the ER is initiated by the formation of a binary complex between the IRES and the 40S ribosomal subunit followed by the assembly of a 48S-like complex at the AUG initiation codon after the association of eukaryotic translation initiation factor 3 (eIF3) and the ternary complex (eIF2•Met-tRNAi•GTP) (figure 1.2c). The 60S subunit is then associated in a GTP-dependent manner to form the 80S complex (Otto and Puglisi, 2004). The resulting polyprotein precursor is co- and post-translationally processed. While the structural proteins and the p7 polypeptide are cleaved by cellular proteases (signalase and signal peptide peptidase, indicated by white and grey triangles in figure 1.1), the non-structural proteins are processed by the two viral proteases NS2-3 protease and NS3-4A serine protease (indicated by red and green triangles in figure 1.1) (Scheel and Rice, 2013).

1.3.3 RNA replication

NS4B and NS5A induce the formation of ER-derived membrane spherules, which in aggregate are called the membranous web. This structure is believed to be the site of HCV RNA replication (Egger et al., 2002; Romero-Brey et al., 2012). Three viral proteins are thought to be involved in RNA replication. The NS3 protein is capable of nucleic-acid binding and 3’ to 5’ translocation coupled to hydrolysis of ATP, as it contains a superfamiliy 2 DExH/D-box helicase domain (Raney et al., 2010). This helicase activity could be important for separation of the nascent and template RNA strands, displacement of RNA-binding proteins or unwinding of local RNA secondary structures. NS5A is a phosphoprotein that consists of three domains. While domains I and II are essential for RNA replication (Tellinghuisen et al., 2004, 2008a), domain III plays a role in virus assembly (Appel et al., 2008; Tellinghuisen et al., 2008b). The

phosphorylation state of NS5A determines the regulation of the balance between RNA replication and downstream processes (Neddermann et al., 2004). The most important viral protein for RNA replication is the RNA-dependent RNA polymerase (RdRp) NS5B. This protein facilitates replication by first synthesising a complementary negative-strand RNA using the genome as a template and subsequently producing a genomic positive-strand RNA from the negative-strand template (figure 1.2d)

1.3.4 Virus assembly and release

Virus assembly and release is tightly regulated and coupled to host cell lipid synthesis (Scheel and Rice, 2013). After processing of the core protein by host cell proteases, the mature protein relocates from the ER membrane to cytoplasmic lipid droplets (cLDs) (Boulant et al., 2006; McLauchlan et al., 2002). This process is facilitated by diacylglycerol acyltransferase-1 (DGAT-1) (Herker et al., 2010). The complete process of nucleocapsid formation has not been fully elucidated yet, but the current model proposes an interaction of core with NS5A either on cLDs or after translocation from the mobile cLDs to the ER (Appel et al., 2008; Miyanari et al., 2007; Shavinskaya et al., 2007). Delivery of HCV genomic RNA to the site of nucleocapsid formation is probably achieved via the close proximity of sites of RNA replication and virion assembly and via NS2-coordinated virion assembly through interactions with glycoproteins, p7, NS3 and NS5A (Jirasko et al., 2010; Popescu et al., 2011). p7 oligomers are believed to form ion channels that prevent the acidification of intracellular compartments and thereby virus inactivation during the release of the viral particle (Wozniak et al., 2010). The E1 and E2 glycoproteins are translocated from the ER by a series of signal and stop-transfer sequences, thereby giving them a type I membrane protein topology. The E1-E2 glycans are subsequently folded, assembled into heterodimers and N-linked sugars are added, before they are trimmed by glycosidases I and II (figure 1.2e and f) (Lavie et al., 2007).

1.4 Therapy

The primary goal of HCV therapy is to cure the infection. A sustained virological response (SVR) is defined as undetectable HCV RNA either 12 weeks (SVR12) or 24

weeks (SVR24) after treatment completion. Until 2011, a combination of pegylated IFN-α and ribavirin was administered for 24-48 weeks to treat HCV infection (Craxì et al., 2011). With this treatment SVR rates of about 40-50% could be achieved in patients infected with genotype 1, while considerably higher SVR rates of up to 80% were achieved in patients infected with genotypes 2, 3, 5, and 6. Intermediate SVR rates were observed in genotype 4 infected patients (Antaki et al., 2010). In 2011, the first wave of first-generation direct-acting antivirals (DAAs) was approved for treatment of HCV genotype 1 infection. Telaprevir and boceprevir are both protease inhibitors, as they target the NS3/4A serine protease. Both drugs must be administered in combination with pegylated interferon (IFN)-α and ribavirin. In phase III trials of these triple drug combinations, SVR rates of 65-75% were achieved, but severe side-effects were still observed (Bacon et al., 2011; Jacobson et al., 2011; Poordad et al., 2011; Zeuzem et al., 2011).

Three new DAAs will be licensed in the European Union in the first half of 2014 for use in combination therapies (Pawlotsky, 2014). Sofosbuvir is a nucleotide analogue inhibitor of the RdRp and has been approved in January 2014. Simperevir is a second-wave first-generation protease inhibitor, which has been approved in May 2014. Daclatasvir is an NS5A inhibitor that will probably be approved in August or September 2014 (Pawlotsky, 2014). All of these show an improved efficacy and tolerability compared to telaprevir and boceprevir (Au and Pockros, 2014).

1.5 HCV genotypes and distribution

HCV has a very high replication rate of an estimated 10^{12} virions per day per person (Neumann et al., 1998). Due to the lack of proofreading activity the RdRp has a high error rate that leads to approximately 10^4 to 10^5 nucleotide exchanges per generation (Drake et al., 1998; Holland et al., 1982). Taken together, this results in the generation of approximately 10^9 viral variants in each infected patient several times per day (Guedj et al., 2010). These viral variants are closely related, but still distinct and are termed the viral quasispecies. Viruses within the same quasispecies show 91-99% similarity in conserved genomic regions, but are distinguishable in the more variable regions such as hypervariable region-1 (HVR-1) and HVR-2. The high variability of HCV facilitates the adaptation to changes in the replication environment like selection pressure imposed by drugs or by the host immune response. Beyond

this sequence diversity within the quasispecies, more substantial differences exist between HCV variants at the population level.

HCV is grouped into seven different genotypes (1-7) with about 30% sequence divergence and multiple subtypes (a,b,c,…) with about 15-20% sequence divergence (Gottwein and Bukh, 2008; Smith et al., 2014). The geographic distribution of these genotypes is quite distinct, with genotype 1 being the most common genotype in America (70%), Japan (75%) and Europe (50-75%). Genotypes 2 and 3 are also common in these areas, while genotypes 4 and 5 are common in Africa, but are also spreading to Europe. Genotypes 3 and 6 are prevalent in South and Southeast Asia. Genotype 7 has recently been isolated from a few patients in Central Africa, but does not have widespread clinical importance yet (Scheel and Rice, 2013).

Genotype distribution does not only differ according to geographical location, but also by transmission route. In Europe infections resulting from infected blood transfusions are predominantly associated with genotype 1b infection, while genotype 1a and 3a are frequently observed in people who inject drugs (PWID) (Esteban et al., 2008).

1.6 Transmission of HCV

HCV is mainly transmitted via exposure to infectious blood or blood products. In the past before the introduction of serological and molecular diagnostic tests, the main risk factor for acquiring HCV was transfusion of blood products. In the late 1980s 2-10% of blood products in developed countries contained HCV (Alter et al., 1981; Colombo et al.; Esteban et al., 1990; Prati, 2006) leading to the infection of most chronic transfusion recipients and patients receiving clotting factor concentrates (Esteban et al., 1989; Mannucci and Tuddenham, 2001; Prati, 2000, 2002). Nowadays, blood products in Europe are very safe and no HCV infections due to the administration of plasma-derived products have been reported since 1994 (Prati, 2006). Nevertheless, blood products remain the major cause of HCV transmission in developing countries without routine HCV diagnostic tests. In developed countries like Germany injection drug use (IDU) has become the main risk factor for HCV infection (Aceijas and Rhodes, 2007; Cornberg et al., 2011; Nelson et al., 2011).

1.7 HCV infection in PWID

Due to their risk behavior of sharing needles and other injection equipment and the

resulting blood-to-blood contact, PWID have become the main risk group for HCV infection. Worldwide an estimated 16 million PWID existed in 2007 (Mathers et al., 2008), while according to the European Monitoring Center for Drugs and Drug Addiction (EMCDDA) and Eurosurveillance an estimated 1.7 million PWID exist in European countries (Esteban et al., 2008; Wiessing, 2005).

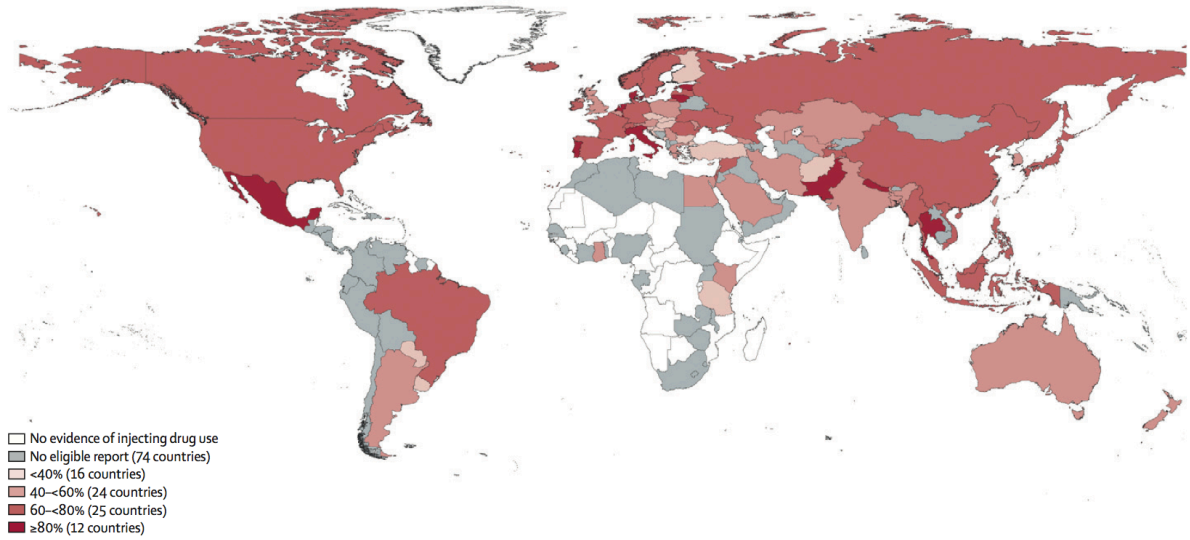

Figure 1.3: Prevalence of HCV antibodies in PWID

Prevalence of anti-HCV antibodies was determined in PWID and used as a marker for contact to HCV. Modified from (Nelson et al., 2011).

Prevalence of HCV infection in PWID ranges widely (figure 1.3), but accounts for 60-80% of prevalent infections in North America and most European countries (Dalgard et al., 2003; Harris et al., 1999; Hutchinson et al., 2006; Nelson et al., 2011; Sutton et al., 2006; Westin et al., 1999). As IDU is a very efficient route of HCV transmission, PWID are rapidly infected after they begin IDU. Factors including age, duration and frequency of IDU, sharing equipment, polydrug use, HCV prevalence among experienced PWID, homelessness and having served a prison sentence also play a role in the swift transmission of HCV in PWID (Garfein et al., 1998; Hagan et al., 2001; Hickman et al., 2007; Judd et al., 2005; Mathei et al., 2006; Villano et al., 1997).

PWID are frequently exposed to different HCV genotypes due to the heterogeneous genotype distribution within this group. Evidence for this can also be found in the existence of intergenotypic HCV recombinants (genotype 1b/2k) in PWID that are rapidly spreading (Kalinina et al., 2002; Moreau et al., 2006). Previous clearance of

HCV infection does not provide sterilizing immunity upon renewed antigen exposure (Aberle et al., 2006; Aitken et al., 2008; Grebely et al., 2006; Mehta et al., 2002; Micallef et al., 2007; Mizukoshi et al., 2008). However, resolved HCV infection is at least associated with partial protection, as the incidence of reinfection is low compared to acquisition of primary HCV infections (Aberle et al., 2006; Mehta et al., 2002; Mizukoshi et al., 2008; Osburn et al., 2010).

In Germany, a recent epidemiological study of PWID revealed that 45.2% of PWID were HCV-RNA positive indicating acute or chronic infection, another 27.9% were anti-HCV positive but HCV-RNA negative consistent with resolved HCV infection, while the remaining 26.9% of PWID were anti-HCV negative although the reported risk behavior suggests that they have been exposed to the HCV (Zimmermann, 2012).

1.8 Immune response against HCV

Both the innate and adaptive arms of the immune response play an important role in the control of HCV infection. During HCV replication the virus is identified as nonself by pattern recognition receptors (PRRs) in the host cell that bind pathogen-associated molecular patterns (PAMPs) within viral products. This PAMP recognition leads to a coordinated activation of the innate and adaptive immune responses.

1.8.1 Innate immune response

The first response during HCV infection is thought to be IFN-β production by infected hepatocytes and plasmacytoid dendritic cells (pDCs). This response is initiated by three PRRs: retinoic acid-inducible gene-I (RIG-I), protein kinase R (PKR) and Toll-like receptor 3 (TLR3).

RIG-I recognizes the HCV 3’ poly (U/UC) sequence, the 5’ triphosphate of the uncapped HCV RNA and short double-stranded (ds) RNA regions (Saito et al., 2008; Uzri and Gehrke, 2009). These HCV PAMPs could be recognized by RIG-I either after viral uncoating when the HCV genome is present in the cytosol or during genome amplification. Binding of HCV RNA to RIG-I induces a conformational change that results in oligomerization and translocation from the cytosol to intracellular membranes, where it ultimately interacts with the mitochondrial antiviral signalling protein (MAVS) (Jiang et al., 2011; Liu et al., 2012; Saito et al., 2007).

The kinase activity of PKR can be activated by binding to the IRES of HCV (Shimoike et al., 2009) and leads to the phosphorylation of the α subunit of eIF2. In addition, PKR binding of HCV RNA also activates a kinase-independent signal transduction cascade also involving MAVS (McAllister and Samuel, 2009).

TLR3 senses dsRNA replication intermediates that accumulate late during HCV replication in endosomes and is expressed in a number of intrahepatic cell types such as hepatocytes and the liver-resident macrophages Kupffer cells (Li et al., 2012; Seki and Brenner, 2008; Wang et al., 2009). Upon ligand binding, TLR3 transmits signals through the adaptor protein Toll/interleukin (IL)-1 receptor (TIR)-domain-containing adaptor-inducing IFN-β (TRIF) (Takeuchi and Akira, 2009).

The activation of both MAVS and TRIF induces a complex assembly of different signalling mediators including tumour necrosis factor (TNF)-receptor associated factor 3 (TRAF3) leading to the activation of downstream effector molecules such as the transcription factors IFN regulatory factor-3 (IRF-3) and nuclear factor-κB (NF-κB) and ultimately the expression of IFN-β, IFN-λ and a small subset of IFN-stimulated genes (ISGs) (Arnaud et al., 2011; Loo and Gale, 2011; Takeuchi and Akira, 2009).

Secretion of IFN-β induces an antiviral state that also extends to surrounding uninfected cells by binding to the type I IFN receptor (IFNAR) and thereby activating the Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) signalling pathway, which results in the induction of more than 300 ISGs with antiviral, antiproliferative and immunomodulatory functions. A few examples include 2'–5' oligoadenylate synthetase 1 (OAS1)/RNAse L, which degrades viral and cellular RNA (Guo et al., 2004), and dsRNA-specific adenosine deaminase 1 (ADAR1), which converts adenosine to inosine in dsRNA leading to the accumulation of mutations during viral replication (Taylor et al., 2005).

Three subtypes of IFN-λ exist: IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B) (Sheppard et al., 2003). All subtypes bind to the same heterodimeric receptor consisting of the IL-28 receptor α-chain and the IL-10 receptor β-chain. Although they bind to a different receptor, type III interferons also activate the JAK-STAT signalling pathway. Furthermore, the pattern of ISGs induced by IFN-λ in liver cells is very similar to the one induced by IFN-α, even though the kinetics of ISG expression are different (Marcello et al., 2006).

Interestingly, several single nucleotide polymorphisms (SNPs) upstream of the *IFNL3* gene have been associated with spontaneous clearance of HCV infection (Thomas et

al., 2009) as well as improved response to IFN-α therapy (Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009), but the mechanism behind this association is still unknown.

HCV has evolved several ways to evade the innate immune responses. The NS3/4A protein can cleave TRIF (Li et al., 2005a) and MAVS (Foy et al., 2005; Li et al., 2005b; Meylan et al., 2005), thereby blocking TLR3, RIG-I and PKR signalling. In addition, HCV core can interfere with JAK/STAT signalling and therefore ISG expression in several ways: It can inhibit STAT1 activation (Lin et al., 2006), induce suppressor of cytokine signalling 3 (SOCS3) (Bode et al., 2003), which is an inhibitor of the JAK/STAT pathway, and inhibit ISG factor 3 binding to IFN-stimulated response elements.

Another cell type that is involved in the innate immune response against HCV are natural killer (NK) cells (reviewed in (Cheent and Khakoo, 2011)), which are more frequently found in the liver than in the blood (Corado et al., 1997). NK cell were originally associated with HCV infection outcome in an immunogenetic study of the killer cell immunoglobulin-like receptor (KIR) genes and their human leukocyte antigen (HLA)-C ligands (Khakoo et al., 2004). In this study KIR2DL3 and its group 1 HLA-C ligand was associated with protection from chronic HCV infection in PWID. The protective effect has been attributed to the lower avidity binding of KIR2DL3 to HLA-C than other inhibitory KIRs and NK cells expressing this inhibitory receptor would therefore have a lower activation threshold allowing for stronger and more rapid antiviral effector functions (Fadda et al., 2010; Moesta et al., 2008). Another possible explanation might be found in a recent study by Thöns *et al.*, who found a negative correlation between the strong inhibitory NK cell receptor NKG2A and KIR2DL3 and the protective effect might thus be mediated by the absence of NKG2A (Thoens et al., 2014).

In addition to genetic studies, NK cells have also been found to be activated in acutely infected patients as indicated by an increased expression of the activating receptor NKG2D compared to healthy subjects (Amadei et al., 2010). This was accompanied by increased production of IFN-γ and cytotoxicity. Even though the cytotoxicity of NK cells was not altered in chronically infected patients, conflicting data exists with regard to cytokine production and in particular IFN-γ production (Ahlenstiel et al., 2010; Amadei et al., 2010; Golden-Mason et al., 2008; Oliviero et al., 2009).

Only limited data exists on the evasion of NK cell responses during HCV infection. It has been suggested that NS5A-containing apoptotic bodies can trigger the production of IL-10 and decrease the IL-12 production by monocytes, which would lead to a downregulation of NKG2D on NK cells (Sène et al., 2010). Crosslinking of CD81 by soluble glycoprotein E2 and subsequent interaction with NK cells also leads to inhibitory signalling in NK cells resulting in reduced cytotoxicity and IFN-γ production *in vitro* (Crotta et al., 2002; Tseng and Klimpel, 2002), but the physiological role of this phenomenon is debatable, as direct exposure to infectious HCV particles did not have the same effect (Crotta et al., 2010; Farag et al., 2011).

1.8.2 Adaptive immune response

1.8.2.1 Humoral immune response

In most acutely infected patients, HCV-specific antibodies (Abs) against epitopes within the structural and non-structural proteins become detectable approximately 7-8 weeks after infection (Pawlotsky, 1999), but most of these have no antiviral activity, as only a small fraction is made up of neutralizing antibodies (nAbs) that can inhibit virus binding, entry or uncoating. Multiple linear, conformational and discontinuous epitopes that are targeted by nAbs have been identified in the HCV envelope glycoproteins E1 and E2 including a “hot-spot” in and adjacent to HVR-1 (Sabo et al., 2011). It has been shown that nAbs target entry at a step after initial binding of HCV, most likely by interrupting HCV E2-CD81 or HCV E2-SR-BI interactions or by inhibiting membrane fusion (Haberstroh et al., 2008). Nevertheless, the role of nAbs during acute HCV infection and particularly viral clearance is incompletely understood.

On the one hand, spontaneous viral clearance without seroconversion has been observed in chimpanzees (Cooper et al., 1999) as well as humans (Post et al., 2004), including immunocompromised patients with primary antibody deficiencies (e.g. hypogammaglobulinaemic patients) (Christie et al., 1997). Taken together these findings seem to indicate that antibody responses against HCV are not necessarily required for viral clearance. Furthermore, numerous studies have suggested that patients with acute-resolving infection lack nAbs, while patients with chronic infection develop these after establishment of persistence (Bartosch et al., 2003; Kaplan et al., 2007; Logvinoff et al., 2004; Netski et al., 2005; Zeisel et al., 2008).

On the other hand, the induction of nAbs during early infection has been associated with spontaneous viral clearance in a study of a single-source genotype 1b outbreak in East Germany caused by a contaminated anti-D immunoglobulin preparation (Pestka et al., 2007). In the same cohort, a delayed development of nAbs was observed in patients with chronic HCV infection. A study by Osburn *et al.*, who found that HCV reinfection and subsequent viral clearance in PWID was associated with the induction of cross-reactive humoral responses, provides further evidence for a beneficial role of nAbs (Osburn et al., 2010). It has also been shown that nAbs drive sequence evolution *in vivo* during acute infection and resolution of infection has been linked to an antibody response that effectively neutralized autologous time-matched viruses (Dowd et al., 2009).

Several mechanisms for the evasion of humoral immune responses have been suggested. Viral escape in epitopes targeted by nAbs might play a role (Farci et al., 1996). In addition, HVR-1 might function as a decoy for nAbs, as it stimulates strong immune responses responsible for viral variant selection that are nevertheless unable to clear the infection (von Hahn et al., 2007). Interaction of the HCV glycoproteins with SR-B1 and high-density lipoprotein (HDL) may protect from nAbs (Logvinoff et al., 2004) and some gylcoproteins on E2 also modulate cell entry and protect from nAbs (Falkowska et al., 2007; Helle et al., 2007). Furthermore, binding of non-neutralizing Abs may prevent the binding of nAbs (Zhang et al., 2007). Recent studies also suggest that HCV can also spread by direct cell-to-cell transfer, thereby circumventing neutralisation (Brimacombe et al., 2011; Timpe et al., 2008; Witteveldt et al., 2009).

1.8.2.2 Cellular immune response

Both $CD4^+$ and $CD8^+$ T cell responses play a critical role during HCV infection. T cell responses against HCV become detectable after a delay of approximately 6-12 weeks.

One indicator of the importance of HCV-specific $CD4^+$ T cells for HCV clearance is the association of certain HLA class II alleles with HCV infection outcome in genetic studies. As $CD4^+$ T cells recognize antigen in the context of major histocompatibility complex (MHC) class II presentation, these cells and their targeting of specific epitopes has been linked to differential infection outcome. The HLA class II alleles most reproducibly correlated with favourable infection outcome in heterogeneous

study cohorts are DRB1*1101 and DQB1*0301, which are in linkage disequilibrium (Hong et al., 2005). However, in a single-source outbreak in Ireland, DRB1*0101, DRB1*0401 and DRB1*15 were associated with viral clearance (McKiernan et al., 2004). Interestingly, most $CD4^+$ T cell epitopes discovered so far are restricted by "protective" HLA alleles, even though these epitopes are in general highly promiscuous and often restricted by multiple HLA class II alleles (reviewed in (Walker, 2010)).

The role of $CD4^+$ T cells on HCV infection outcome can at least partly be attributed to the fact that they provide support for $CD8^+$ T cells during the acute phase of infection. Loss of helper activity has been linked to poor $CD8^+$ T cell function in humans (Francavilla et al., 2004; Kaplan et al., 2008; Urbani et al., 2006). *In vivo* experiments in chimpanzees corroborate these findings, as antibody-mediated depletion of $CD4^+$ T cells in previously protected animals led to loss of viral control and emergence of escape variants in MHC class I epitopes (Grakoui et al., 2003).

In addition, studies in patients with acute HCV infection found strong, multi-specific and sustained HCV-specific $CD4^+$ T cell responses in patients that subsequently resolved infection (Day et al., 2002; Diepolder et al., 1995, 1997; Gerlach et al., 1999; Missale et al., 1996; Schulze zur Wiesch et al., 2005). These $CD4^+$ T cells show better proliferation and IL-2, IFN-γ and TNF-α production. Recently, one particular $CD4^+$ T cell subset has been associated with viral clearance, namely $CD161^{high}CCR6^+CD26^+CD4^+$ T cells that express IL-17A as well as IL-21 and Th17-lineage-specific transcription factors (Kared et al., 2013) (figure 1.4).

When broad $CD4^+$ T cell responses can be detected during the acute phase in patients who go on to develop chronic infection, these cells later undergo rapid exhaustion with sequential loss of IL-2 production, proliferation and IFN-γ secretion (Gerlach et al., 1999; Semmo et al., 2005) as well as elevated expression of inhibitory receptors such as T cell immunoglobulin domain and mucindomain 3 (TIM-3), programmed death 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4) (Raziorrouh et al., 2011).

$CD8^+$ T cells are critical for viral clearance. Similar to MHC class II alleles, several MHC class I alleles have also been linked to HCV infection outcome. In particular, HLA-A*03, HLA-B*27, HLA-B*57 and HLA-C*01 have been associated with viral clearance of genotype 1 infection (Kim et al., 2011; Kuniholm et al., 2010; McKiernan et al., 2004), while HLA-B*08, HLA-B*18 and HLA-C*04 have been linked to viral

persistence (Kim et al., 2011; McKiernan et al., 2004; Thio et al., 2002). The protective effects of HLA-A*03, HLA-B*27 and HLA-B*57 have been linked to immunodominant $CD8^+$ T cell epitopes (Fitzmaurice et al., 2011; Neumann-Haefelin et al., 2010; Oniangue-Ndza et al., 2011).

The appearance of vigorous HCV-specific $CD8^+$ T cell responses in the liver and the peripheral blood targeting multiple epitopes has been temporally linked with initial control of acute phase viremia and a peak in serum transaminases (Cooper et al., 1999; Cox et al., 2005a; Grüner et al., 2000; Lechner et al., 2000; Thimme et al., 2001, 2002). Importantly, $CD8^+$ T cells have also been shown to play a critical role in viral control *in vivo*. In an HCV-infected chimpanzee antibody-mediated depletion of $CD8^+$ T cells led to loss of viral control, until the HCV-specific $CD8^+$ T cell responses recovered (Shoukry et al., 2003).

In general, $CD8^+$ T cells can eliminate virus-infected hepatocytes in two different ways: non-cytolytic, cytokine-mediated inhibition of replication, which does not require the destruction of the infected cell, and perforin/granzyme-mediated or surface death receptor-mediated cytolytic destruction of infected cells. In *in vitro* experiments virus-specific $CD8^+$ T cells have been shown to exert strong antiviral effector functions primarily via IFN-γ production and only to a lesser degree via direct cytolytic effector functions (Jo et al., 2009). This finding has also been confirmed in chimpanzees, where viral clearance in the acute phase can occur without elevated transaminase levels and only minimal histological evidence for liver cell injury, but with detectable IFN-γ messenger RNA (mRNA) levels (Su et al., 2002).

During the acute phase of HCV infection HCV-specific $CD8^+$ T cells exhibit a stunned phenotype that is characterised by impaired proliferation, cytotoxicity and IFN-γ production irrespective of eventual infection outcome (Lechner et al., 2000; Thimme et al., 2001; Urbani et al., 2006). In addition, these $CD8^+$ T cells also exhibit elevated surface expression of PD-1 (Kasprowicz et al., 2008). However, this increased PD-1 expression can be transient in acute HCV infection and shows a similar pattern as the activation marker CD38 (Kasprowicz et al., 2008; Shin et al., 2013). PD-1 can therefore probably be considered as a marker of activation rather than exhaustion on HCV-specific $CD8^+$ T cells during acute infection. In patients who later resolve HCV infection PD-1 is eventually downregulated and the antiapoptotic marker B cell lymphoma-2 (Bcl-2) and the IL-7 receptor α-chain CD127, which also serves as a

memory marker, get upregulated instead (figure 1.4) (Shin et al., 2013; Thimme et al., 2001; Urbani et al., 2006).

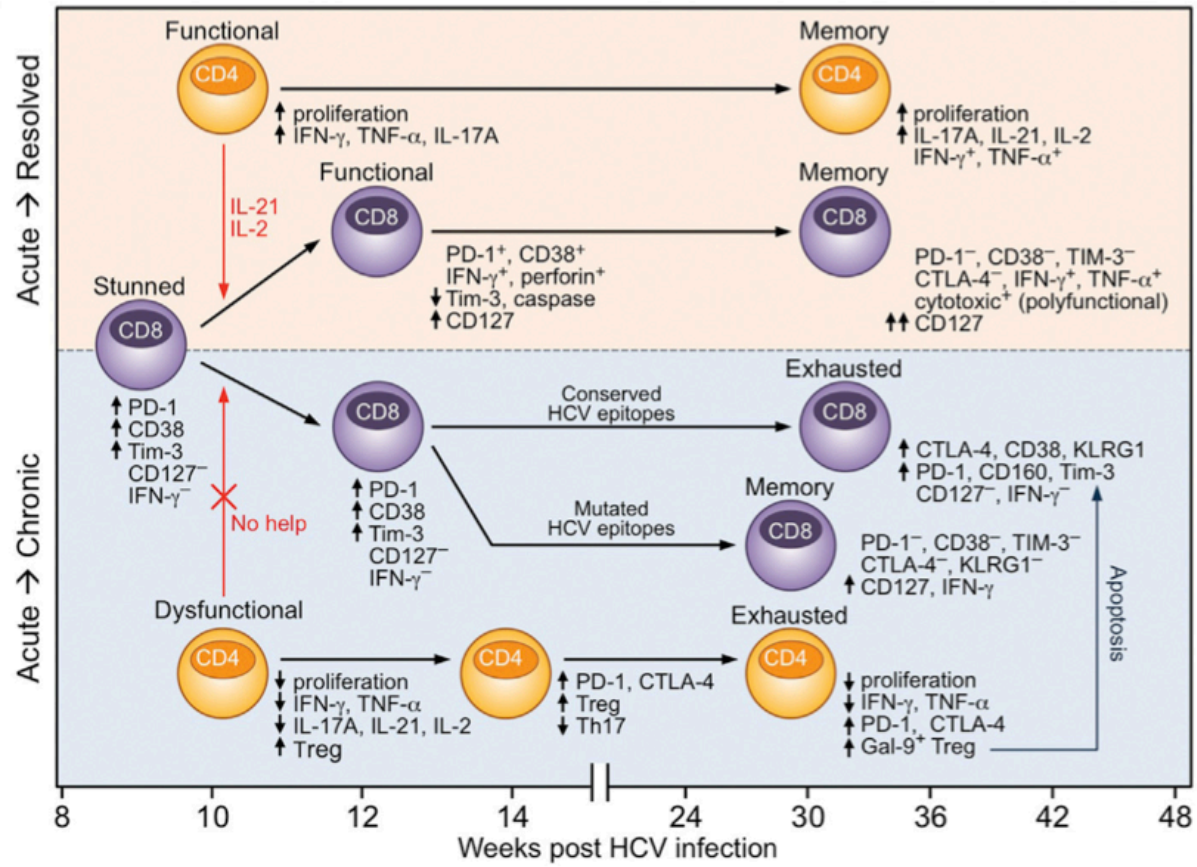

Figure 1.4: Phenotype of CD4$^+$ and CD8$^+$ T cells during acute, chronic and resolved HCV infection

A strong and maintained CD4$^+$ T cell response appears to be a critical factor for the outcome of acute HCV infection. In its presence, HCV-specific CD8$^+$ T cell populations with an initially ‘‘stunned’’ phenotype acquire multiple effector functions (top). In the absence or loss of a strong CD4$^+$ T cell response, CD8$^+$ T cells develop exhausted phenotypes, which are attributed to chronic antigen-specific stimulation. Those CD8$^+$ T cells that target HCV escape variants remain functional with a memory phenotype in the chronic phase of infection. Treg: regulatory T cell. Modified from (Park and Rehermann, 2014).

In most patients with chronic infection, HCV-specific CD8$^+$ T cells are also present, but are unable to control the infection. Several factors play a role in the failure of the CD8$^+$ T cell response. One of these factors is the dysfunction of a large fraction of HCV-specific CD8$^+$ T cells during chronic infection, which is characterised by their inability to secrete antiviral cytokines such as IFN-γ and their inability to proliferate in response to antigen stimulation (Nakamoto et al., 2009; Radziewicz et al., 2007, 2008; Spangenberg et al., 2005; Wedemeyer et al., 2002). These cells also express increased levels of the inhibitory receptor PD-1 (Golden-Mason et al., 2007;

Nakamoto et al., 2008; Penna et al., 2007; Radziewicz et al., 2007, 2008) and low levels of CD127 (Bengsch et al., 2007; Golden-Mason et al., 2007; Radziewicz et al., 2007). Very high levels of PD-1 expression have also been associated with caspase-9 dependent apoptosis of $CD8^+$ T cells in patients that develop chronic infection, thereby resulting in the depletion of HCV-specific $CD8^+$ T cells (Radziewicz et al., 2008). In addition, other inhibitory receptors like Tim-3, CD160, 2B4 (CD244) and CTLA-4 as well as the T cell senescence marker killer-cell-lectin-like receptor G1 (KLRG1) are also upregulated (Bengsch et al., 2010; Golden-Mason et al., 2009; Nakamoto et al., 2009). The function of HCV-specific $CD8^+$ T cells co-expressing multiple inhibitory receptors could only partially be restored *in vitro* by blockade of PD-1 (Bengsch et al., 2010) and full restoration of function necessitated the blockade of more than one receptor (Nakamoto et al., 2009). The mechanisms causing this exhaustion of HCV-specific $CD8^+$ T cells are incompletely understood, but contributing factors probably include the lack of $CD4^+$ T cell help mentioned above as well as the action of regulatory T cells or cytokines.

Increased numbers of $CD4^+CD25^+$ T cells have been found in chronically HCV-infected patients compared to patients with resolved infection and healthy controls (Boettler et al., 2005; Cabrera et al., 2004; Sugimoto et al., 2003). These regulatory T cells can suppress both the IFN-γ production and the proliferation of virus-specific $CD8^+$ T cells *in vitro* in a cell-cell contact dependent manner (Sugimoto et al., 2003). Virus-specific regulatory $CD8^+$ T cells also exist. These cells have been found in the liver of HCV infected patients and express high levels of the inhibitory cytokine IL-10 (Abel et al., 2006).

The liver microenvironment probably also contributes to the dysfunction of HCV-specific $CD8^+$ T cells. Several mechanisms that bias immune responses in the liver towards tolerance have been proposed. These include antigen-presenting cells, such as liver dendritic cell subsets as well as liver sinusoidal dendritic cells and hepatic stellate cells, that might induce immune suppression via different mechanisms including the secretion of inhibitory cytokines like IL-10 and transforming growth factor (TGF)-β, the expression of inhibitory molecules like programmed death-ligand 1 (PD-L1) on Kupffer cells, pDCs, liver sinusoidal endothelial cells (LSECs), stellate cells and hepatocytes or the failure to provide sufficient co-stimulation (reviewed in (Crispe, 2011; Protzer et al., 2012; Thomson and Knolle, 2010)). Hepatocytes have also been shown to function as antigen-presenting cells leading to the activation of

dysfunctional $CD8^+$ T cells (Bowen et al., 2004) and $CD8^+$ T cells that are prone to apoptosis in a Bcl-2-interacting mediator of death (Bim)-dependent manner in murine models (Holz et al., 2008). The individual contributions of these factors to HCV-specific $CD8^+$ T cell dysfunction still need to be clarified.

Not all HCV-specific $CD8^+$ T cells in chronically infected patients have an exhausted phenotype, but functional $CD8^+$ T cells usually target epitopes that harbour escape mutations (Bengsch et al., 2010), thereby implicating viral escape as another major factor responsible for the failure of the HCV-specific $CD8^+$ T cell response.

As mentioned above, HCV exists as a viral quasispecies within one patient due to both the high replication rate of HCV and its error-prone RdRp, which facilitates the selection of escape mutations. Evidence for viral escape has been found both in infected chimpanzees (Erickson et al., 2001; Weiner et al., 1995) and patients (Chang et al., 1997) and the emergence of escape mutations has been observed during acute infection. These escape mutations during acute infection were mainly observed in patients who went on to develop chronic infection, but not in patients who resolved infection spontaneously (Cox et al., 2005a, 2005b; Tester et al., 2005; Timm et al., 2004).

Many escape mutations are also associated with varying degrees of fitness costs, i.e. the replicative capacity of the virus is impaired to some extent (Neumann-Haefelin et al., 2008a; Ruhl et al., 2012; Salloum et al., 2008; Uebelhoer et al., 2008). As a result, reversions back to the prototype sequence are often observed in the absence of immune pressure either due to transmission of the virus to a patients with a different HLA allele background (Ray et al., 2005; Timm et al., 2004) or due to pregnancy-induced immune tolerance (Honegger et al., 2013).

Different molecular mechanisms of viral escape have been proposed. Mutations in the MHC binding anchors of the epitope, usually position 2 and the terminal amino acid, abrogate binding to the MHC molecule, while mutation in the centre of the epitope tend to interfere with the recognition by the T cell receptor (TCR) (Söderholm et al., 2006). Mutations in the flanking region may additionally interfere with proteasomal processing (Kimura et al., 2005; Seifert et al., 2004; Timm et al., 2004). In addition, the lack of $CD4^+$ T cell help has been implicated in viral escape in $CD8^+$ T cell epitopes (Grakoui et al., 2003). Another study in chimpanzees has associated limited TCR diversity with the emergence of viral escape mutations, while a broader

TCR repertoire may possibly prevent escape mutations due to cross-reactivity with more sequence variants of the same epitope (Meyer-Olson et al., 2004).

1.9 Aim of the study

$CD8^+$ T cells are an integral part of the immune against HCV. A multitude of factors can influence whether the HCV-specific $CD8^+$ T cell response is able to spontaneously clear the infection. The viral genotype also plays an important role in determining infection outcome. The sequence diversity between different genotypes can lead to the presentation of different epitopes to $CD8^+$ T cells. This has already been shown to be important, as HLA-B*27 is only associated with viral clearance in genotype 1, but not genotype 3, which has been linked to an immunodominant $CD8^+$ T cell epitope that is not conserved between the two genotypes. Other known differences between genotype 1 and 3 include their different responses to IFN-α therapy, with genotype 3 being associated with a more favourable outcome, and the association of the *IFNL3* genotype with infection outcome and response to therapy.

Even though these differences are known, only limited data exists about $CD8^+$ T cells during genotype 3 infection in general and the factors influencing their ability to clear HCV infection.

Consequently, we wanted to compare what influence different host factors like the HLA background and the *IFNL3* genotype have on the outcome of HCV infection with either genotype 1 or 3 in particular with regard to the HCV-specific $CD8^+$ T cell response. Furthermore, we wanted to test, whether both genotype-specific variants of an epitope can be recognised in one patient. As a last step, we also wanted to see whether the infecting viral genotype influences the exhaustion and deletion of HCV-specific $CD8^+$ T cells.

2 Materials

2.1 Chemicals, enzymes and reagents

BD FACS Clean Solution	Becton Dickinson (BD)
BD FACSFlow Sheath Fluid	BD
BD FACSRinse Solution	BD
BD FACS Shutdown Solution	BD
Biocoll Separating Solution	Biochrome
Dimethylsulfoxid (DMSO)	Roth
Deoxynucleotide Triphosphate (dNTP) Mix	myBudget
Dulbecco's Phosphate-Buffered Saline, 1X (PBS)	Gibco
Ethanol	Sigma-Aldrich
GoTaq Flexi Deoxyribonucleic acid (DNA) Polymerase	Promega
IC Fixation Buffer	eBioscience
Isopropanol	Sigma-Aldrich
Permeabilization Buffer (10X)	eBioscience

2.2 Cell culture media and additives

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1M	PAA
Brefeldin A (BFA)	Sigma Aldrich
Fetal bovine serum (FBS)	Biochrome
Penicillin/Streptomycin (100X)	PAA
Roswell Park Memorial Institute (RPMI) 1640 + L-Glutamin	PAA
IL-2	Roche
CD28/CD49d Costimulatory Reagent	BD

2.3 Composition of cell culture media

R10	RPMI 1640
	10% FBS
	10 mM HEPES
	100 U/ml penicillin
	100 µg/ml streptomycin
Freezing medium	FBS
	10% DMSO

2.4 Commercial kits

LightCycler FastStart DNA Master^{PLUS} HybProbe polymearse chain reaction (PCR) Kit	Roche
LightSNiP Typing Assay rs12979860 *Homo sapiens*	TIB MOLBIOL
QIAamp Viral RNA Mini Kit	Qiagen
QIAamp DNA Blood Mini Kit	Qiagen
Qiagen OneStep reverse transciptase (RT)-PCR Kit	Qiagen

2.5 Antibodies and other staining reagents for flow cytometry

All conjugated antibodies were stored at 4 °C and all unconjugated antibodies were stored at -20 °C. Fixable Viability Dyes were aliquoted and stored at -80 °C to void multiple freeze/thaw cycles. If not otherwise indicated, all antibodies were mouse anti-human.

Table 2.1: Antibodies and other staining reagents

specificity	fluorochrome	clone	dilution	concentration	company
$Bim_{S/EL/L}$ (rat anti-human)	unconjugated	3C5	1:10		Enzo Life Sciences
CD3	Peridinin chlorophyll protein (PerCP)-Cyanine (Cy)5.5	OKT3	1:1000	0.05mg/ml	
CD4	Phycoerythrin (PE)	RPA-T4	1:1000	0.2 mg/ml	eBioscience
CD4	Allophycocyanin (APC)-eFluor780	RPA-T4	1:500	0.1 mg/ml	eBioscience
CD8	eFluor450	RPA-T8	1:500		eBioscience
CD8	PerCP-Cy5.5	RPA-T8	1:400	0.05 mg/ml	eBioscience
CD8	APC	RPA-T8	1:400	0.012 mg/ml	eBioscience
CD38	APC	HIT2	1:50		BD
CD127	PE-Cy7	HIL-7R-M21	1:20		BD
Fixable Viability Dye	eFluor780	n.a.	1:5000		eBioscience
Fixable Viability Dye	eFluor506	n.a.	1:1000		eBioscience
IgG2a (goat anti-rat)	Fluorescein isothiocyanate (FITC)	polyclonal	1:200	1 mg/ml	Bethyl Laboratories
IFN-γ	FITC	4S.B3	1:1000	0.5 mg/ml	eBioscience
IFN-γ	PE-Cy7	4S.B3	1:1500	0.2 mg/ml	eBioscience
Vβ 1	PE	BL37.2	1:200		Beckman Coulter
Vβ 2	FITC	MPB2D5	1:100		Beckman Coulter
Vβ 3	FITC	CH92	1:20		Beckman Coulter
Vβ 4	FITC	WJF24	1:20		Beckman Coulter

specificity	fluorochrome	clone	dilution	concentration	company
Vβ 5.1	APC	LC4	1:100	0.1 mg/ml	eBioscience
Vβ 5.2	PE	36213	1:10		Beckman Coulter
Vβ 5.3	APC	3D11	1:100	0.1 mg/ml	eBioscience
Vβ 7.1	PE	ZOE	1:20		Beckman Coulter
Vβ 7.2	PE	ZIZOU4	1:100		Beckman Coulter
Vβ 8	eFluor450	JR-2	1:500	0.05 mg/ml	eBioscience
Vβ 9	APC	AMKB1-2	1:100	0.05 mg/ml	eBioscience
Vβ 11	APC	C21	1:20		Beckman Coulter
Vβ 12	FITC	VER2.32.1	1:20		Beckman Coulter
Vβ 13.1	FITC	H131	1:100	0.2 mg/ml	eBioscience
Vβ 13.2	PE	H132	1:100	0.0025 mg/ml	eBioscience
Vβ 13.6	PE	JU74.3	1:20		Beckman Coulter
Vβ 14	PE	CAS1.1.3	1:20		Beckman Coulter
Vβ 16	FITC	TAMAYA1.2	1:100		Beckman Coulter
Vβ 17	PE	E17.5F3.15.13	1:20		Beckman Coulter
Vβ 18	PE	BA62.6	1:20		Beckman Coulter
Vβ 20	FITC	ELL1.4	1:10		Beckman Coulter
Vβ 21.3	FITC	IG125	1:10		Beckman Coulter
Vβ 22	FITC	IMMU 546	1:50		Beckman Coulter
Vβ 23	PerCP-eFluor710	AF23	1:50	0.1 mg/ml	eBioscience

n.a.: not applicable

TCR Vβ antibodies were grouped into 9 pools for flow cytometric analysis of the Vβ repertoire. Vβ segments and corresponding antibodies used in these pools, which cover about 70% of the normal human TCR Vβ repertoire of $CD3^+$ lymphocytes, are shown in table 2.2.

Table 2.2: TCR Vβ pools

pool ID	Vβ* (#)	fluorochrome
pool 1	Vβ 3 (TRBV28)	FITC
	Vβ 7.1 (TRBV4-1, TRBV4-2, TRBV4-3)	PE
	Vβ 5.3 (TRBV5-5)	APC
pool 2	Vβ 16 (TRBV14)	FITC
	Vβ 17 (TRBV19)	PE
	Vβ 9 (TRBV3-1)	APC
pool 3	Vβ 20 (TRBV30)	FITC
	Vβ 18 (TRBV18)	PE
	Vβ 5.1 (TRBV5-1)	APC
pool 4	Vβ 13.1 (TRBV6-5, TRBV6-6, TRBV6-9)	FITC
	Vβ 13.6 (TRBV6-6)	PE
	Vβ 8 (TRBV12-3, TRBV12-4)	eFluor450
pool 5	Vβ 2 (TRBV20-1)	FITC
	Vβ 5.2 (TRBV5-6)	PE
pool 6	Vβ 21.3 (TRBV11-2)	FITC
	Vβ 1 (TRBV9)	PE
	Vβ 23 (TRBV13)	PerCP-eFluor710
pool 7	Vβ 22 (TRBV2)	FITC
	Vβ 14 (TRBV27)	PE
	Vβ 11 (TRBV25-1)	APC
pool 8	Vβ 4 (TRBV29-1)	FITC
	Vβ 13.2 (TRBV6-2)	PE
pool 9	Vβ 12 (TRBV10-3)	FITC
	Vβ 7.2 (TRBV4-3)	PE

* nomenclature according to Wei *et al.* (Wei et al., 1994)

IMGT nomecalture (Lefranc, 2003; Lefranc et al., 1999)

2.6 MHC class I dextramers

MHC class I dextramer consist of a dextran polymer backbone carrying an optimized number of MHC class I molecules as well as fluorochrome molecules. All MHC class I dextramers were ordered from Immundex and were stored at 4 °C in the dark.

Table 2.3: MHC class I dextramers

ID	position	protein	genotype (GT)	HLA-restriction	sequence	fluorochrome
p1	1436	NS3	1a/1b/3a	HLA-A*01	ATDALMTGY	PE
p11	1406	NS3	1a	HLA-A*02	KLVALGINAV	PE
p20	2841	NS5B	1a/1b	HLA-B*27	ARMILMTHF	FITC and PE
p24	1073	NS3	1a/1b	HLA-A*02	CINGVCWTV	PE
p47	2594	NS5B	1a	HLA-A*02	ALYDVVTKL	PE
p67	1273	NS3	1a/1b	HLA-A*02	GVDPNIRTGV	PE
p99	1373	NS3	1a/1b/3a	HLA-B*51	IPFYGKAI	PE
p384	840	NS2	3a	HLA-B*27	GRLIWWNQY	PE
p387	942	NS2	3a	HLA-B*27	GRWFNTYLY	APC
CMV	495	pp65	n.a.	HLA-A*02	NLVPMVATV	PE
Flu	58	matrix	n.a.	HLA-A*02	GILGFVFTL	PE

2.7 Oligonucleotides

All primers were ordered from biomers.net. Lyophilised primers were reconstituted in RNAse free water at a concentration of 10 pmol/µl and stored at -20 °C.

Table 2.4: Oligonucleotides used for PCR

ID	GT	position	sequence	amplified fragment (PCR round)
HCV1a-2e-F	1a	2531	CGTCTGCTCCTGCTTGTGG	NS2 (1)
HCV1a-3a-F	1a	2718	ATGTGGCCTCTCCTCCTGC	NS2 (2)
HCV1a-3b-R	1a	3390	ACCCCTTGGAGACCATTCC	NS2 (2)
HCV1a-3c-R	1a	3746	TCCRAATGGAGACCAAGC	NS2 (1)
HCV1a-3b-F	1a	2919	TGGRTTCCCCCCCTCAACG	NS3 (1)
HCV1a-3c-F	1a	3271	TCCRAATGGAGACCAAGC	NS3 (2)
HCV1a-4d-R	1a	5306	ACCCAGGTGCTCGTGACG	NS3 (2)
HCV1a-4e-R	1a	5519	GGGCCCTTCTGCTTGAACTGC	NS3 (1)
HCV1a-7b-F	1a	7952	CCACATCAACTCCGTGTGG	NS5B (1)
HCV1a-7d-F	1a	8625	GCTATGACCAGGTACTCCG	NS5B (2)
HCV1a-7e-R-int	1a	9325	CCTGCAGCAAGCAGGAGTAGGC	NS5B (2)
HCV1a-3'prime end	1a	9384	TAAGAGGCCGGAGTGTTTAYCC	NS5B (1)
HCV1b-2d-F	1b	2415	CTCCAYCAGAACATCGTGG	NS2 (1)
HCV1b-2e-F	1b	2650	TGTTCTTCTGTGCYGCCTGG	NS2 (2)
HCV1b-3b-R	1b	3400	ATGGGCGCRAGGAGTCGC	NS2 (2)
HCV1b-3c-R	1b	3717	CCAARTAAAGGTCCGAGCTGCC	NS2 (1)
HCV1b-3274-F	1b	3274	GACATGGAGACCAARRTCATCA	NS3 (1)
HCV1b-3353-F	1b	3353	CCGAAGGGGRARGGAGAT	NS3 (2)
HCV1b-5502-R	1b	5502	TGYTCGGCGAGCTGCATYCC	NS3 (2)
HCV1b-5638-R	1b	5638	TCCCGCTGATGAARTTCC	NS3 (1)

ID	GT	position	sequence	amplified fragment (PCR round)
HCV1b-sv365s	1b	7254	GACTACGTCCCTCCMGTGGTAC	NS5B (1)
HCV1b-sv394s	1b	7412	GTCGGCCGTCGACAGCGGCACRGCRAC	NS5B (2)
HCV1b-sv347as	1b	8960	AGGTAGGTCAAGTGGYTCAATG	NS5B (2)
HCV1b-sv350as	1b	9006	GTGGAGTGTAAATGCGCTAAG	NS5B (1)
HCV3a-2367-F	3a	2367	CCTTGCTCYTTCACGCCCATG	NS2 (1)
HCV3a-2756-F	3a	2756	CAACGGGCGTAYGCTTGG	NS2 (2)
HCV3a-3974-R	3a	3974	TCAGARAARGATGGRGACC	NS2 (2)
HCV3a-4055-R	3a	4055	CYTTYGTGCTCTTACCRCTGC	NS2 (1)
HCV3a-3256-F	3a	3256	CTAGCAGTGGCCACTGAACC	NS3 (1)
HCV3a-3341-F	3a	3341	ATATTCTTTGCGGGCTGC	NS3 (2)
HCV3a-5439-R	3a	5439	CACCTCYTTRTCTGGAACG	NS3 (2)
HCV3a-5452-R	3a	5452	TATTGTTGRTACARCACCTC	NS3 (1)

2.8 Peptides

All HCV-specific peptides were ordered from peptides&elephants or EMC at 70% purity. The lyophilised peptides were reconstituted with DMSO at a concentration of 20 mg/ml and stored at -80 °C. Working solutions were prepared at a concentration of 1 mg/ml by dilution of the stock solutions with RPMI medium without any additives. Working solutions were stored at -20 °C.

Table 2.5: HCV-specific peptides contained in HLA-matched peptide pools

HLA-type	ID	aa position	protein	genotype	sequence
A*01	p1	1436	NS3	1a, 1b, 3a	ATDALMTGY
A*02	p11	1406	NS3	1a	KLVALGINAV
	p14	1131	NS3	1a, 1b	YLVTRHADV
	p24	1073	NS3	1a, 1b	CINGVCWTV
	p42	1406	NS3	1b	KLSGLGLNAV
	p47	2594	NS5B	1a	ALYDVVTKL
	p67	1273	NS3	1a, 1b	GVDPNIRTGV
	p96	1987	NS5A	1a	VLSDFTTWL
	p119	2479	NS5B	1b	VLDDHYWDV
A*03	p126	2510	NS5B	1b	KLTPPHSAK
	p193	1858	NS4B	1a, 1b	GVAGALVAFK
	p211	43	Core	1a, 1b, 3a	RLGVRATRK
	p212	51	Core	1a, 1b, 3a	KTSERSQPR
	p229	2587	NS5B	1a, 1b	RVCEKMALY
A*03/A*11	p16	1261	NS3	1a, 1b	TLGFGAYMSK
	p34	1635	NS3	1a, 1b	VTLTHPITK
	p38	1227	NS3	1a, 1b	HLHAPTGSGK
	p59	1227	NS3	3a	YLHAPTGSGK
A*11	p17	1265	NS3	1a, 1b	GAYMSKAHGV
	p154	2	Core	1a, 1b	STNPKPQKK
	p210	1	Core	1a, 1b	MSTNPKPQK

HLA-type	ID	aa position	protein	genotype	sequence
A*11	p220	621	E2	1a	TINYTIFK
A*23/A*24	p156	838	NS2	1a	YISWCLWWL
A*24	p12	1031	NS3	1b	AYSQQTRGL
	p13	1100	NS3	1a, 1b, 3a	MYTNVDQDL
A*24	p65	1292	NS3	1a, 1b, 3a	TYSTYGKFL
	p155	674	E2	1a, 1b	ILPCSFTTL
A*25	p142	1758	NS4B	1a, 1b	EAFWAKHMW
	p143	1758	NS4B	1a, 1b variant	ETFWAKHMW
	p189	1744	NS4B	1a	EVITPAVQTNW
	p223	831	NS2	1a	LSPYYKRYIS
	p231	2818	NS5B	1a, 1b, 3a	TARHTPVNSW
A*26	p30	1534	NS3	1a, 1b, 3a	ETTVRLRAY
	p187	2416	NS5A/B	1a, 1b	DVVCCSMSY
A*26/B*18	p144	1582	NS3	1b	DNFPYLVAY
A*29	p221	790	p7	1a	FYGMWPLLL
	p222	827	NS2	1a	MALTLSPY
A*68	p50	1175	NS3	1a, 1b	HAVGIFRAAV
B*07	p114	1111	NS3	1b	WPAPSGARSL
	p148	2836	NS5B	1b	APTLWARMVL
	p149	2000	NS5A	1b	LPRLPGVPF
	p184	41	Core	1a, 1b, 3a	GPRLGVRAT
	p214	111	Core	1a, 1b, 3a	DPRRRSRNL
B*08	p3	1395	NS3	1a, 1b, 3a	HSKKKCDEL
	p61	1611	NS3	1a, 1b, 3a	LIRLKPTL
B*13	p32	1627	NS3	1a, 1b	RLGAVQNEV
	p33	1627	NS3	3a	RLGPVQNEI
B*15	p39	1265	NS3	3a	GSFMSRAYGI
	p117	2450	NS5B	1b	LLRHHNMVY
	p146	2466	NS5B	1b	SQRQKKVTF
B*27	p20	2841	NS5B	1a, 1b	ARMILMTHF
	p56	1492	NS3	3a	GRGRIGTYRY
	p58	1492	NS3	1a	GRGKPGIYRF
	p185	2820	NS5B	1a, 1b, 3a	ARHTPVNSW
B*35	p22	1359	NS3	1a, 1b, 3a	HPNIEEVAL
	p186	2163	NS5A	1a, 1b	EPEPDVAVL
	p191	2000	NS5A	1b variant	LPKLPGVPF
	p215	169	Core	1a, 1b, 3a	LPGCSFSIF
	p216	234	E1	1a	NASRCWVAM
	p219	589	E2	1a	HPEATYSRC
	p225	1695	NS4A	1a, 1b	IPDREVLY
B*37	p190	1966	NS4B/5A	1a	SECCTPCSGSW
B*38	p192	2218	NS5A	1a	NHDSPDAEL
	p227	1941	NS4B	1a	AARVTAIL
	p230	2793	NS5B	1a	HDGAGKRKYYL
B*40	p73	530	E2	1a	GENDTDVFVL
	p77	2152	NS5A	1a	HEYPVGSQL

HLA-type	ID	aa position	protein	genotype	sequence
B*40	p79	2336	NS5A	1a	TESTVSTAL
	p83	2266	NS5A	1a, 1b	REISVPAEIL
	p87	1871	NS4B	1a, 1b	GEVPSTEDL
	p91	654	E2	1a, 1b	LEDRDRSEL
	p93	453	E2	1a	PERLASCRPL
	p106	2142	NS5A	1a	REEVSFRVGL
	p107	2152	NS5A	1a	HEYPVGSQL
	p109	530	E2	1b	GENETDVLLL
	p110	1871	NS4B	1b	GEMPSTEDL
	p111	2142	NS5A	1b	REEVTFQVGL
B*44	p213	88	Core	1b	NEGLGWAGW
B*51	p99	1373	NS3	1a, 1b, 3a	IPFYGKAI
	p217	489	E2	1a	YPPKPCGI
B*57	p19	2629	NS5B	1a, 1b	KSKKTPMGF
	p21	541	E2	1a	NTRPPLGNW
B*58	p218	521	E2	1a, 1b	RSGAPTYSW
	p224	1596	NS3	1a, 1b, 3a	RAQAPPPSW
	p228	1968	NS4B	1a	CTTPCSGSW

Table 2.6: Predicted HLA-B*27 restricted HCV-specific peptides

HLA-type	ID	aa position	protein	genotype	sequence
B*27	p20	2841	NS5B	1a, 1b	ARMILMTHF
	p56	1492	NS3	3a	GRGRIGTYRY
	p57	1492	NS3	1b	GRGRRGIYRF
	p58	1492	NS3	1a	GRGKPGIYRF
	p185	2820	NS5B	1a, 1b, 3a	ARHTPVNSW
	p375	39	Core	1a, 1b, 3a	RRGPRLGVR
	p376	61	Core	1a, 1b, 3a	RRQPIPKAR
	p377	73	Core	1b	GRAWAQPGY
	p378	113	Core	1a, 1b, 3a	RRRSRNLGKV
	p379	316	E1	1a, 1b, 3a	HRMAWDMMM
	p380	547	E2	3a	GRWFGCTWM
	p381	729	E2	3a	ARVCVALWL
	p382	780	p7	1a	GRWVPGAAY
	p383	840	NS2	1b	ARLIWWLQY
	p384	840	NS2	3a	GRLIWWNQY
	p385	869	NS2	1a	GRDAVILLM
	p386	919	NS2	1b	IRACMLVRK
	p387	942	NS2	3a	GRWFNTYLY
	p388	1004	NS2	3a	ARLGREVLL
	p389	1144	NS3	1a, 1b	RRGDSRGSL
	p390	1487	NS3	1a	RRGRTGRGK
	p391	1487	NS3	1b, 3a	RRGRTGRGR
	p392	1492	NS3	3a	GRGRLGTYR

HLA-type	ID	aa position	protein	genotype	sequence
B*27	p393	1494	NS3	3a	GRLGTYRYV
	p394	1499	NS3	1a	YRFVAPGER
	p395	1499	NS3	1b	YRFVTPGER
	p396	1499	NS3	3a	YRYVAPGER
	p397	1958	NS4B	1a	RRLHQWISS
	p398	2094	NS5A	3a	RRVGDFHYI
	p399	2188	NS5A	1a, 1b, 3a	RRLARGSPP
	p400	2279	NS5A	1a	RRFAPALPI
	p401	2328	NS5A	3a	RRKRTIQLD
	p402	2500	NS5B	3a	ARMLTIEEA
	p403	2619	NS5B	3a	QRVERLLKM
	p404	2813	NS5B	1a, 1b, 3a	ARAAWETAR
	p405	2841	NS5B	3a	VRMVMMTHF
	p406	2884	NS5B	1a, 1b	QRLHGLSAF
	p407	2917	NS5B	1a, 3a	LRAWRHRAR
	p408	2920	NS5B	1a	WRHRARSVR
	p409	2920	NS5B	3a	WRHRARAVR
	p410	2922	NS5B	1a	HRARSVRAR
	p411	2922	NS5B	1b	HRARSVRAK
	p412	2922	NS5B	3a	HRARAVRAK
	p413	2924	NS5B	1a	ARSVRARLL
	p414	2924	NS5B	1b	ARSVRAKLL
	p415	2924	NS5B	3a	ARAVRAKLI
	p416	2929	NS5B	1a	ARLLSRGGR
	p417	2936	NS5B	1a	GRAAICGKY
	p418	2936	NS5B	1b	GRAATCGKY
	p419	2985	NS5B	1b	SRARPRWFM
	p420	2985	NS5B	3a	SRARTRYLL
	p421	2989	NS5B	1a	PRWFWFCLL
	p422	2989	NS5B	1b	PRWFMLCLL
	p423	2989	NS5B	3a	TRYLLLCLL

Table 2.7: HCV-specific peptides used for cross-reactivity studies

HLA-type	ID	aa position	protein	genotype	sequence
A*02	p11	1406	NS3	1a	KLVALGINAV
	p24	1073	NS3	1a, 1b	CINGVCWTV
	p25	1073	NS3	3a	TVGGVMWTV
	p42	1406	NS3	1b	KLSGLGLNAV
	p43	1406	NS3	1b variant	KLSALGLNAV
	p47	2594	NS5B	1a	ALYDVVTKL
	p67	1273	NS3	1a, 1b	GVDPNIRTGV
	p290	1273	NS3	escape variant	GVEPNIRTGV
	p291	1073	NS3	escape variant	CINGVCWSI
	p292	1406	NS3	escape variant	KLAGMGLNAV

HLA-type	ID	aa position	protein	genotype	sequence
A*02	p293	1406	NS3	escape variant	KLVTLGINAV
	p295	1406	NS3	escape variant	KLVALGVNAV
	p296	2594	NS5B	escape variant	ALYDVVSKL
	p297	2594	NS5B	escape variant	ALYDVVGKL
B*13	p32	1627	NS3	1a, 1b	RLGAVQNEV
	p33	1627	NS3	3a	RLGPVQNEI
	p366	1627	NS3	escape variant	RLGPVQNET
	p367	1627	NS3	escape variant	RLGPVQNEF
	p368	1627	NS3	escape variant	RLGSVQNEI
B*15	p146	2466	NS5B	1b	SQRQKKVTF
	p373	2466	NS5B	1a	CQRQKKVTF
	p374	2466	NS5B	3a	SQRQRKVTF
B*27	p383	840	NS2	1b	ARLIWWLQY
	p384	840	NS2	3a	GRLIWWNQY
	p387	942	NS2	3a	GRWFNTYLY
	p426	840	NS2	1a	SWCLWWLQY
	p427	942	NS2	1a	GALTGTYVY
	p428	942	NS2	1b	AALTGTYVY
	p430	840	NS2	escape variant	SRIIWWNQY
	p431	840	NS2	escape variant	ARLMWWNQY
	p432	840	NS2	3a variant	GRLMWWNQY
	p433	942	NS2	escape variant	GKWFNTYLY
	p434	942	NS2	escape variant	GRWCNTYLY
B*51	p98	1373	NS3	escape variant	VPFYGKAI
	p99	1373	NS3	1a, 1b, 3a	IPFYGKAI
	p294	1373	NS3	escape variant	IPFYGKAL
B*57	p19	2629	NS5B	1a	KSKKTPMGF
	p21	541	E2	1a	NTRPPLGNW
	p371	2629	NS5B	1b	KSKKCPMGF
	p372	2629	NS5B	3a	TSKKTPLGF
	p424	541	E2	1b	NTRPPQGNW
	p425	541	E2	3a	SLRPPSGRW

CEF Control Peptide Pool was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. The lyophilised peptide pool was reconstituted with DMSO at a concentration of 20 mg/ml per peptide and stored at -80 °C. A working solution was prepared at a concentration of 1 mg/ml by diluting the stock solution with RPMI medium without any additives. The working solution was stored at -20 °C.

Table 2.8: Peptides contained in CEF Control Peptide Pool

HLA-type	virus	peptide sequence
A*01	Influenza A	VSDGGPNLY
A*01	Influenza A	CTELKLSDY
A*02	**Influenza M**	**GILGFVFTL**
A*02	Influenza A	FMYSDFHFI
A*02	EBV LMP2A	CLGGLLTMV
A*02	EBV BMLF1	GLCTLVAML
A*02:01	**HCMV pp65**	**NLVPMVATV**
AA68	Influenza NP	KTGGPIYKR
A*03	Influenza NP	RVLSFIKGTK
A*03	Influenza A	ILRGSVAHK
A*03	EBV	RVRAYTYSK
A*03	EBV	RLRAEAQVK
A*03, A*11, A*60:81	Influenza M	SIIPSGPLK
A*11	EBV EBNA 4NP	AVFDRKSDAK
A*11	EBV	IVTDFSVIK
A*11	EBV	ATIGTAMYK
A*24	EBV RTA	DYCNVLNKEF
B*07	Influenza NP	LPFDKTTVM
B*07	EBV	RPPIFIRRL
B*08	Influenza NP	ELRSRYWAI
B*08	EBV B2LF-1	RAKFKQLL
B*08	EBV EBNA 3A	FLRGRAYGL
B*08	EBV EBNA 3	QAKWRLQTL
B*18	HCMV	SDEEEAIVAYTL
B*27	Influenza NP	SRYWAIRTR
B*27	Influenza M	ASCMGLIY
B*27	EBV EBNA 3C	RRIYDLIEL

Peptides indicated in bold were also purchased as single peptides from peptides&elephants.

2.9 Patients

Blood samples from PWID were collected from the ward for inpatient detoxification treatment of drug addicts or the clinic for opioid maintenance treatment (OMT) at the Department of Addictive Behaviour and Addiction Medicine, Rhine State Hospital Essen, Hospital of the University of Duisburg-Essen. In total 363 blood samples were collected. 40 samples were seronegative for HCV antibody (by anti-HCV CMIA from Abbott), while 323 patients were HCV antibody positive, including 91 HCV-RNA negative patients, 120 with genotype 1, 98 with genotype 3 and 14 infected with other genotypes. HCV viral load was determined with the Versant HCV RNA 3.0 (bDNA) assay with a detection limit 615 international units (IU)/ml and further tested in a qualitative Versant assay (TMA) with a detection limit of 10 IU/ml. Patients without detectable HCV-RNA who had previously received antiviral therapy for HCV were excluded from this study. Written informed consent was obtained from all patients and the study was approved by the local ethics committee.

In addition, plasma samples from eleven HLA-B*27 positive individuals with chronic HCV genotype 3a infection who presented to the University Hospital of Freiburg were included in the sequence analysis of HLA-B*27 positive patients. Samples were kindly provided by Christoph Neumann-Haefelin, Department of Medicine II, University of Freiburg, Freiburg, Germany.

2.10 Consumables and equipment

Cell culture flasks (T25, T75)	Greiner Bio-One
Cell culture plates, flat bottom (6-, 12-, 24-, 48-, 96-well)	Greiner Bio-One
Centrifuge 5415 R	Eppendorf
Centrifuge 5424	Eppendorf
Cryo tubes, 2.0 mL	Greiner Bio-One
FACS Calibur	Becton Dickinson
FACS Canto II	Becton Dickinson
KX-21N	Sysmex
Leucosep™ tubes, 50 mL	Greiner Bio-One
Lightcycler 2.0	Roche
Mastercycler gradient	Eppendorf
Mastercycler personal	Eppendorf
MJ Mini	BIO RAD
Megafuge 1.0R	Heraeus
Megafuge 40R	Thermo Scientific
Microscope Primo Vert	Zeiss
Microscope TMS	Nikon
Mr. Frosty freezing container	Thermo Scientific
Navios	Beckman Coulter
NanoDrop	Thermo Scientific
Polystyrene round-bottom tubes, 5 mL	BD Falcon
Thermomixer comfort	Eppendorf
Thermostat plus	Eppendorf

2.11 Software and webpages

Allele Frequency Net Database	http://www.allelefrequencies.net
CodonCode Aligner 4.2.5	CodonCode Corporation
FlowJo 10.0.7	Tree Star Inc.
Geneious 7.0.6	Biomatters Ltd.
Graph Pad Prism 5	GraphPad Software, Inc.
HCV sequence database (Kuiken et al., 2005)	http://hcv.lanl.gov/content/index
MHC-I Binding Prediction Tool	http://tools.immuneepitope.org/mhci
Microsoft Office 2008 for Mac	Microsoft Corporation

3 Methods

3.1 Immunological methods

3.1.1 Isolation and cryopreservtion of perihpherial blood mononuclear cells (PBMCs)

PBMCs were isolated from peripheral blood samples via Ficoll density centrifugation (Böyum, 1968). Briefly, 15 ml of Biocoll separating solution were pipetted into a 50 ml Leucosep tube and centrifuged for 1 min at 491 x *g* in order to allow the Biocoll to pass through the filter inside the tube. Subsequently, 30 ml of blood were added to the tube and centrifuged for 10 min at 1055 x *g*. Due to the different densities of the cell types present in blood, separation into different layers occurs during centrifugation. Red blood cells and granulocytes aggregate at the bottom of the tube below the filter, while lymphocytes, monocytes and platelets are not dense enough to penetrate the Biocoll layer and accumulate at the interphase above the filter. The layer containing the lymphocytes was then transferred into a new 50 ml Falcon tube and centrifuged at 872 x *g* for 8 min. The cells were washed twice with 40 ml PBS in order to remove platelets. During the second washing step 200 µl of PBMCs were removed. The cells were pelleted and frozen at -20°C for later DNA extraction (see 3.2.1). Afterwards cells were resuspended in freezing medium and placed into a Mr. Frosty freezing container, which was placed at -80 °C. After one day the PBMCs were transferred into liquid nitrogen.

3.1.2 Thawing of PBMCs

PBMCs were thawed in a 37 °C water bath and transferred into a 15 ml Falcon tube containing 9 ml of PBS. The cells were then centrifuged at 706 x *g* for 6 min. Afterwards the supernatant was discarded and the cells were washed with 10 ml of PBS. The total cell number was determined using the KX-21N cell counter.

3.1.3 Expansion of HCV-specific $CD8^+$ T cells for intracellular cytokine staining (ICS)

After thawing PBMCs were resuspended at a concentration of $2x10^6$ cells/ml in R10 medium containing 25 U/ml recombinant IL-2 and 0.1 µg/ml anti-CD28 and anti-

CD49d. Depending on the experiment, the cells were then stimulated with different HCV-specific peptides (1 µg/ml per peptide). As a positive control one well containing 2 x 10^6 cells was stimulated with CEF Peptide Control Pool. PBMCs were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 7 days half the total volume of the culture medium containing IL-2 was added.

3.1.4 Analysis of the HCV-specific $CD8^+$ T cell response against HLA-matched peptide pools by ICS

On day 10 after stimulation with HLA-matched peptide pools (see table 2.5) aliquots of 1-2 x 10^5 cells were transferred into 96 well round bottom plates, washed twice with R10 medium and then restimulated with the same peptide pools (10 µg/ml per peptide) for 4 h in the presence of BFA (100 ng/ml). One well was left unstimulated (no peptide) as a negative control. The BFA was used to prevent transport of intracellular vesicles continaing e.g. IFN-γ from the ER to the cell surface, thereby facilitating the later intracellular detection of IFN-γ. Subsequently, the PBMCs were pelleted at 491 x *g* for 5 min and washed once with PBS containing 1% FBS (PBS/FBS). The cells were then stained with antibodies against the surface antigens CD4 and CD8 (CD4 PE and CD8 APC) in a total volume of 100 µl of PBS/FBS for 10 min at 4 °C. After another washing step with PBS/FBS, the PBMCs were fixed for 15 min at 4 °C using IC fixation buffer. Following two washing steps with 1x Permeabilization Buffer, the cells were intracellularly stained for IFN-γ (IFN-γ FITC) in 100 µl of 1x Permeabilization Buffer for 20 min at 4 °C. After another washing step with PBS the cells were resuspended in 200 µl of PBS, transferred into 5 ml round-bottom tubes and acquired either on a FACSCalibur running CellQuest software or on a Navios. Analysis of expression patterns was done using FlowJo.

$CD8^+$ T cell responses were counted as positive, if the percentage of $CD8^+IFN\text{-}\gamma^+$ T cells was at least twice as high in the peptide stimulated sample as in the unstimulated control sample after subtraction of the background. If positive responses were detected, the experiment was repeated the following day using the corresponding single peptides contained in the positive HLA-matched pool to determine against which peptide the $CD8^+$ T cell response was directed.

3.1.5 Analysis of the CD8$^+$ T cell response against putative novel HLA-B*27 restricted epitopes by ICS

In order to discover potential new HLA-B*27 restricted epitopes, the consensus sequence of all GT1a, GT1b or GT3a sequences available in the HCV sequence database (see table 4.1) were entered into the MHC-I Binding Predictions Tool (http://tools.immuneepitope.org/mhci). The MHC class I binding predictions were made using the IEDB analysis resource Consensus tool (Kim et al., 2012) which combines predictions from ANN aka NetMHC (3.4) (Lundegaard et al., 2008; Nielsen et al., 2003), SMM (Peters and Sette, 2005) and Comblib (Sidney et al., 2008). All 9mers that had a percentile rank score ≤1 for binding to the HLA-B*27:05 molecule were ordered from peptides&elephants and used for further analysis. In addition, known HLA-B*27 restricted peptides were also included in the analysis.

After thawing (see 3.1.2) and expansion (see 3.1.3) of PBMCs from HLA-B*27 positive PWID, the cells were stimulated with HLA-B*27 restricted peptides (see table 2.6) for 10 days. ICS staining for IFN-γ was done essentially as described above (see 3.1.4), but before staining for surface molecules the PBMCs were stained with Fixable Viability Dye eFluor780 for 15 min at 4 °C to exclude dead cells. In addition, CD3 PerCP-Cy5.5 was included in the surface stain master mix to exclude non-T cells from further analysis steps. All samples were acquired on a FACSCanto II running FACSDiva software and further analysed using FlowJo.

3.1.6 *Ex vivo* analysis of HLA-B*27 restricted HCV-specific CD8$^+$ T cells via MHC-class I dextramer staining

PBMCs from 25 HLA-B*27 positive PWID were stained directly after thawing and after 7 days of peptide-specific expansion to determine the frequency of HCV-specific CD8$^+$ T cells directed against three different HLA-B*27 restricted epitopes. For this purpose, 2-3x10^6 cells per patient were transferred into one well on a 96-well round bottom plate and washed once with PBS. The cells were first stained with Fixable Viability Dye eFluor506 for 15 min at 4 °C to exclude dead cells. Subsequently, the PBMCs were stained with 10 µl of MHC class dextramer (p20 labelled with FITC, p384 labelled with PE and p387 labelled with APC) that had been diluted 1:5 with PBS/FBS for 10 min at 4 °C. After another washing step antibodies against CD3 labelled with PerCP-Cy5.5, CD4 labelled with APC-eFluor780, CD8 labelled with eFluor450 and the memory marker CD127 labelled with PE-Cy7 were added and the

cells were incubated for 15 min at 4 °C. After fixation the cells were washed and then acquired on a FACSCanto II running FACSDiva software and further analysed using FlowJo. Dextramer responses were counted as positive if they constituted at least 0.004% of the total $CD8^+$ T cell population and a distinct population could be identified.

3.1.7 Analysis of the cross-reactivity of $CD8^+$ T cells against several sequence variants of the same epitope

After expansion of PBMCs with different variants of the same epitope in different wells for 10 days, the cells were separately restimulated with the peptide used for expansion as well as the variants of the same epitope that should be analysed for cross-reactivity for 4 h. Subsequently, an ICS for the production of IFN-γ was performed as described in 3.1.5.

3.1.8 Analysis of the TCR Vβ repertoire in HCV-specific $CD8^+$ T cells

In order to analyse the TCR Vβ chain usage of HCV-specific $CD8^+$ T cells, PBMCs were expanded in the presence of HCV-specific peptides. After 11 days 1-2 x 10^5 cells were restimulated with the same peptides (10 µg/ml) for 4 h in the presence of BFA. One well was left unstimulated (no peptide) as a negative control. Afterwards the cells were washed with PBS and stained with Fixable Viability Dye eFluor506 for 15 min at 4 °C to exclude dead cells. After another washing step, the PBMCs were stained with antibodies against all surface antigens (the 9 TCR Vβ pools (table 2.2), CD4 APC-eFluor780 and CD8 eFluor450 (for pool 4 CD8 APC was used instead)) for 10 min at 4 °C. After fixation and permeabilization, the cells were additionally stained with antibodies against IFN-γ labelled with PE-Cy7. All samples were acquired on a FACSCanto II running FACSDiva software and further analysed using FlowJo.

3.1.9 Phenotypical analysis of HCV-specific $CD8^+$ T cells via dextramer staining

HCV-specific $CD8^+$ T cells from 24 PWID who exhibited HCV-specific $CD8^+$ T cell responses during the screen with HLA-matched peptides (see 3.1.4) were stained directly after thawing and after 7 days of peptide-specific expansion. For each patient

stainings were performed that included antibodies against Bim in addition to antibodies against CD8, CD38 and MHC class I dextramer. Thus, after thawing $1x10^6$ cells were transferred into two wells on a 96-well round bottom plate and washed once with PBS/FBS. The PBMCs were then stained with 10 µl of MHC class dextramer that had been diluted 1:10 with PBS/FBS for 10 min at room temperature in the dark. After another washing step antibodies against CD8 labelled with PerCP-Cy5.5 and the activation marker CD38 labelled with APC were added and the cells were incubated for 15 min at 4 °C. Following fixation and permeabilization, the cells were stained with 10 µl of antibody directed against the pro-apoptotic marker Bim, which was diluted 1:10 with Permeabilization buffer, for 30 min at room temperature in the dark. Subsequently, an antibody against IgG2a labelled with FITC was added as a secondary antibody against the Bim antibody to be able to detect it by flow cytometry. The PBMCs were again incubated for 30 min at room temperature in the dark and then acquired either on a FACSCalibur running CellQuest software or on a Navios and further analysed using FlowJo.

3.2 Molecular biology methods

3.2.1 Isolation of DNA from PBMCs

Genomic DNA was extracted from PBMCs using the QIAamp Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In short, pelleted PBMCs were thawed (aliquoted during washing steps in 3.1.1) and resuspended in 200 µl of PBS. Subsequently, 20 µl of QIAGEN protease and 200 µl of AL buffer were added and the mixture was pulse vortexed for 15 s. After 10 min of incubation at 56 °C, 200 µl of ethanol were added, the whole sample was transferred into a QIAamp Mini Spin Column and centrifuged for 1 min at 5939 x*g*. The flow through was discarded and the column was placed in a new 2 ml collection tube before adding 500 µl of AW1 buffer and centrifuging for 1 min at 5939 x*g.* After discarding the flow through, the column was washed with 500 µl of AW2 buffer and centrifuged for 3 min at 18188 x*g*. The column was then placed in a new collection tube and centrifuged for 1 min at 18188 x*g* in order to remove excess liquid. DNA was eluted from the column by addition of 50 µl of distilled water and incubation for 1 min at room temperature. The last step was repeated once so that DNA was eluted in a total

volume of 100 µl. The DNA concentration was determined via NanoDrop measurement.

3.2.2 Analysis of the *IFNL3* genotype

IFNL3 rs12979860 genotyping was performed using the LightSNiP Typing Assay rs12979860 *Homo sapiens* and the LightCycler FastStart DNA Master^{PLUS} HybProbe PCR Kit on a LightCycler 2.0 Instrument. The amplification protocol consisted of an initial denaturation step of 95°C for 10 min followed by 45 cycles of 95°C for 10s, 60°C for 10s and 72°C for 15s and subsequent melting curve analysis. Analysis of the *IFNL3* genotype distribution between different HCV infection outcomes was performed using Fisher's exact test.

3.2.3 Analysis of the HLA-type

HLA-A and HLA-B typing at two-digits resolution-level was performed using sequence-specific primers (PCR-SSP) methodology or alternatively using sequence-specific oligonucleotides (LABType methodology, both provided by One Lambda Inc., Canoga Park, CA, USA) at the Institute for Transfusion Medicine, University Hospital Essen, Essen, Germany (Oudshoorn et al., 2007).

Analysis of the HLA type distribution between different HCV infection outcomes was performed using Fisher's exact test or Chi-square test (depending on group size). Significant or borderline significant results were further tested via logistic regression analysis to avoid false positive results at the Department of Bioinformatics, University of Duisburg-Essen, Essen, Germany.

3.2.4 Analysis of the epitope-specific avidities to the HLA-B*27:05 molecule

Peptide binding studies were performed at the Sanquin Company in Amsterdam, the Netherlands. Binding of different peptides to the HLA-B*27:05 MHC class I-molecules was tested via UV-induced peptide-exchange reactions whose efficiency were determined by HLA class I ELISA. In brief, a UV-labile peptide was used to stabilize the HLA class I molecules to form the whole complex. Through UV irradiation at 366 nm in the presence of the peptide of interest, the UV-labile peptide is cleaved and can be exchanged for the peptide of interest yielding an HLA class I complex with the

peptide of choice. If a peptide with a low binding affinity is used for the exchange, the complex will quickly disintegrate, while a peptide with a high binding affinity will stabilize the complex (Toebes et al., 2006). This procedure was followed by an HLA class I ELISA, that can only detect intact HLA class I complexes, as the capture of the HLA class I molecule is performed via the α-chain of the HLA class I molecule, while the detection antibody is directed towards the β2-microglobulin. A peptide that binds with high affinity to HLA-B*27:05 was used as positive control.

3.2.5 Isolation of viral RNA from plasma samples

HCV viral RNA was extracted from PWID plasma samples using the QIAamp Viral RNA Mini Kit. In short, 140 µl of patient plasma were mixed with 560 µl of AVL buffer/carrier RNA that had been preheated to 80 °C. The mixture was incubated at room temperature for 10 min before 560 µl of ethanol were added. After 15 s of vortexing, 630 µl were transferred into a QIAamp Mini column and centrifuged at 5939 x*g* for 1 min. The flow through was discarded and the previous step was repeated for the remaining 630 µl. After each of the following steps a new collection tube was used. The column was washed twice, once with 500 µl of AW1 buffer and once with 500 µl of AW2 buffer before eluting the RNA into a new Eppendorf tube with 60 µl of AVE buffer. The RNA was then stored in two aliquots of 30 µl at -80 °C until further use.

3.2.6 Qualitative RT-PCR

To analyse the autologous viral sequence of chronically HCV infected PWID, the NS2, NS3 or NS5B fragments of different patients was amplified using a combined RT- and nested PCR approach. For this purpose 5 µl of viral RNA isolated from patient plasma (see 3.2.5) were used for transcription into complementary DNA (cDNA) and then directly amplified using the Qiagen OneStep RT-PCR Kit according to the manufacturer's instructions. The master mix used for RT-PCR is shown in table 3.1 and the amplification protocols in table 3.2 and table 3.3. The reaction was performed in a total volume of 25 µl. Primer combinations for all PCR steps are shown in table 2.4.

Table 3.1: Master mix for RT-PCR

component	volume
RNAse-free water	11 µl
5x OneStep RT-PCR Buffer	5 µl
dNTP Mix, 10 mM each	1 µl
F-primer	1 µl
R-primer	1 µl
QIAGEN OneStep RT-PCR Enzyme Mix (Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotStarTaq DNA Polymerase)	1 µl
RNA template	5 µl

Table 3.2: Amplification protocol for RT-PCR of NS2 and NS3 fragments

step	temperature	duration	cycles
reverse transcription	50 °C	30.0 min	1
initial PCR activation step	95 °C	15.0 min	1
denaturation	94 °C	0.5 min	35
annealing	54 °C	0.5 min	
elongation	72 °C	2.5 min	
final elongation	72 °C	20.0 min	1

Table 3.3: Amplification protocol for RT-PCR of NS5B fragment

step	temperature	duration	cycles
reverse transcription	50 °C	30.0 min	1
initial PCR activation step	95 °C	15.0 min	1
denaturation	94 °C	0.5 min	35
annealing	54 °C	0.5 min	
elongation	72 °C	2 min	
final elongation	72 °C	20.0 min	1

The resulting PCR products were then further amplified in a second nested PCR step. The master mix and amplification protocol are shown in table 3.4, 3.5 and 3.6, respectively. The reaction was performed in a total volume of 50 µl.

Table 3.4: Master mix for nested PCR

component	volume
aqua dest.	29 µl
5x Green GoTaq Flexi Buffer (containing mM $MgCL_2$)	14 µl
dNTP Mix, 10 mM each	1 µl
F-primer	1 µl
R-primer	1 µl
GoTaq Flexi DNA Polymerase	1 µl
PCR product	3 µl

Table 3.5: Amplification protocol for nested PCR of NS2 and NS3 fragments

step	temperature	duration	cycles
initial denaturation	95 °C	2.0 min	1
denaturation	94 °C	0.5 min	
annealing	54 °C	0.5 min	35
elongation	72 °C	2.0 min	
final elongation	72 °C	20.0 min	1

Table 3.6: Amplification protocol for nested PCR of NS5B fragment

step	temperature	duration	cycles
initial denaturation	95 °C	2.0 min	1
denaturation	94 °C	0.5 min	
annealing	56 °C	0.5 min	35
elongation	72 °C	1.0 min	
final elongation	72 °C	20.0 min	1

To verify the amplification of fragments of the correct size, 2 µl of PCR product were applied to a 1.2% agarose gel and gel electrophoresis was performed at 100 V for 30 min.

3.2.7 HCV sequence analysis

HCV PCR fragments (3 µl) were mixed with 9 µl of aqua dest. and 2 µl of either forward, reverse or one of the internal primers and send to LGC genomics for sequencing. HCV sequence analysis was performed using CodonCode Aligner or Geneious.

4 Results

4.1 PWID cohort

Injection drug use is the most common risk factor for HCV infection in Germany and other developed countries (Cornberg et al., 2011). Here we work with a cohort of 363 PWID who were recruited from the ward for inpatient detoxification treatment of drug addicts or the clinic for OMT at the Department of Addictive Behaviour and Addiction Medicine, Rhine State Hospital Essen, Hospital of the University of Duisburg-Essen. Approximately 90% of patients were HCV antibody positive, including 232 patients (64%) with detectable HCV RNA and 91 RNA negative patients (25%) (table 4.1).
While there were no significant differences in either the viral load or the ratio between male and female patients, the age of the subjects differed considerably between the subgroups. Seronegative patients were significantly younger than either chronically GT1 infected patients or patients with undetectable RNA levels.

Table 4.1: PWID patient characteristics

	total	GT 1	GT 3	other GT	RNA neg	SN	p-values
number of subjects (% of total)	363 (100.0)	120 (33.1)	98 (27.0)	14 (3.9)	91 (25.1)	40 (11.0)	-
age in years, mean (SD)	37.5 (8.7)	39.3 (10.1)	36.4 (7.6)	39.9 (9.1)	38.0 (7.3)	32.5 (8.0)	GT1 vs. SN: *** RNA neg vs. SN: **
male/female	277/86	90/30	71/27	12/2	73/18	31/9	-
viral load in kIU/ml, median	451.9	675.2	236.8	506.5	-	-	-
viral load in kIU/ml, interquartile range	75.2-1536.0	83.2-1867.0	64.3-1284.0	120.4-975.1	-	-	-

GT: genotype, SN: seronegative, SD: standard deviation, IU: international units

The distribution of HCV genotypes was quite heterogeneous with GT1 and GT3 being the predominant infecting genotypes (33.1% and 27.0% of the total cohort, respectively) (table 4.1). When the cohort was further subdivided into male and female patients within the chronically infected subgroup, it became evident that HCV genotype distribution was very similar between male and female infected patients (figure 4.1). No significant differences between the sexes could be found for the individual genotypes (p-values were calculated using Fisher's exact test), even though there was a slight trend towards more GT1a infection in male patients and

more GT1b and GT3a infection in female patients. Interestingly, GT4 infection occurred only in male patients.

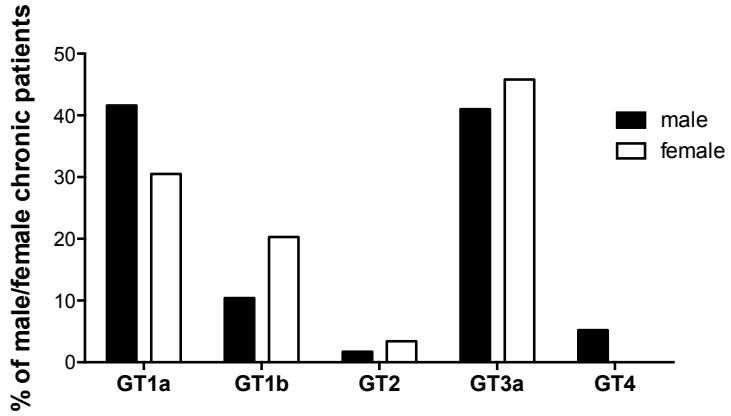

Figure 4.1: Genotype distribution

Genotype distribution within the chronic PWID subgroup according to sex.

4.2 Genetic host factors and their influence on HCV infection outcome

As host genetic factors are known to play an important role during viral infections in general and also HCV infection in particular, we wanted to investigate what role genetic host factors play in determining the outcome of HCV infection. Here, we were particularly interested in potential differences between GT1 and GT3 infections.

4.2.1 *IFNL3* genotype

Genome-wide association studies have identified single nucleotide polymorphisms on chromosome 19q13.13 near the *IFNL3* gene (formerly known as *IL28B*), which encodes for IFN-λ3, that are strongly associated with both spontaneous clearance of HCV (Thomas et al., 2009) as well as improved response to antiviral therapy with IFN-α during GT1 infection (Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009). The precise mechanism behind these associations has not yet been completely elucidated.

As a first step, it was tested whether the association of the *IFNL3* rs12979860 C/C genotype with spontaneous clearance could be reproduced in the PWID cohort. For this purpose, the *IFNL3* rs12979860 genotype was determined in a subgroup of 249 seropositive PWID, including 73 patients with undetectable RNA levels and 176 chronically infected patients. The “protective” *IFNL3* rs12979860 C/C genotype was

significantly less frequent in GT1 infected patients compared to patients with resolved HCV infection (46.6% vs. 57.5%, p=0.0056; figure 4.2). Subjects infected with GT3 showed an intermediate *IFNL3* C/C genotype frequency of 50%.

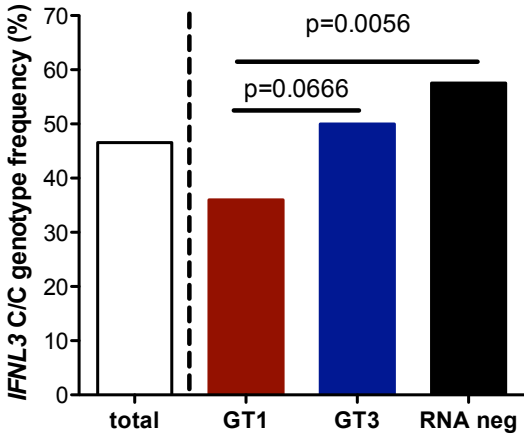

Figure 4.2: Frequency of the *IFNL3* C/C genotype according to infection outcome

The frequency of the rs12979860 C/C genotype in the total cohort and in different subgroups. p-values were calculated using Fisher's exact test.

4.2.2 HLA class I alleles

As a second host factor, we examined the influence of the HLA class I type A and B genotype on the outcome of HCV infection in the PWID cohort. DNA from PWID was typed at two-digit resolution level and compared to data from the general German population that was obtained from the German bone marrow registry via allelefrequencies.net (population: Germany, n=11407) (Gonzalez-Galarza et al., 2011).

No data was available about the infecting genotype in patients with spontaneously resolved HCV infection, but the frequency of GT1 and GT3 infection was very similar in the overall cohort (see table 4.1). Therefore, the following comparisons were performed based on the assumption that a similar frequency of both genotypes in the chronic subgroup of the cohort would also indicate a similar frequency of patients who had resolved either GT1 or GT3 infection in the HCV RNA negative subgroup of the cohort.

Table 4.2: HLA class I allele frequencies of PWID cohort

HLA-allele	German population[+]	PWID cohort	GT1		GT3		RNA neg		GT1 vs. RNA neg			GT3 vs. RNA neg		
	%	%	#	%	#	%	#	%	p-value*	OR	95%CI	p-value	OR	95%CI
A*01	15.0	13.9	40	16.7	21	10.7	25	13.7	0.409	1.26	0.73-2.16	0.369	0.75	0.41-1.40
A*02	29.2	33.7	78	32.5	74	37.8	56	30.8	0.705	1.08	0.72-1.64	0.153	1.36	0.89-2.09
A*03	15.5	14.4	37	15.4	24	12.2	28	15.4	0.993	1.00	0.59-1.71	0.376	0.77	0.43-1.38
A*11	5.1	5.0	10	4.2	14	7.1	7	3.8	0.868	1.09	0.41-2.91	0.162	1.92	0.76-4.88
A*23	2.4	1.6	4	1.7	3	1.5	3	1.6	1.000	1.01	0.22-4.58	1.000	0.93	0.18-4.65
A*24	9.5	8.1	15	6.3	18	9.2	17	9.3	0.235	0.65	0.31-1.33	0.958	0.98	0.49-1.97
A*25	2.3	2.9	10	4.2	6	3.1	2	1.1	0.077	3.91	0.85-18.08	0.287	2.84	0.57-14.27
A*26	3.4	2.6	4	1.7	8	4.1	4	2.2	0.731	0.75	0.19-3.06	0.384	1.89	0.56-6.40
A*29	2.7	2.1	8	3.3	0	0.0	5	2.7	0.730	1.22	0.39-3.80	**0.025**	**0.00**	n.a.
A*30	2.3	2.6	5	2.1	5	2.6	6	3.3	0.438	0.62	0.19-2.08	0.666	0.77	0.23-2.56
A*31	2.3	1.9	3	1.3	3	1.5	6	3.3	0.183	0.37	0.09-1.51	0.323	0.46	0.11-1.85
A*32	3.8	3.9	12	5.0	6	3.1	6	3.3	0.391	1.54	0.57-4.19	0.896	0.93	0.29-2.93
A*33	1.3	1.5	1	0.4	5	2.6	3	1.6	0.320	0.25	0.03-2.42	0.725	1.56	0.37-6.63
A*66	0.4	0.6	1	0.4	1	0.5	2	1.1	0.580	0.38	0.03-4.19	0.611	0.46	0.04-5.13
A*68	4.1	5.2	12	5.0	8	4.1	12	6.6	0.484	0.75	0.33-1.7	0.276	0.60	0.24-1.51
B*07	13.1	13.4	35	14.6	27	13.8	21	11.5	0.361	1.31	0.73-2.34	0.514	1.22	0.67-2.25
B*08	10.0	9.1	30	12.5	15	7.7	11	6.0	**0.027**	**2.22**	**1.08-4.56**	0.537	1.29	0.58-2.88
B*12	0.0	0.2	0	0.0	0	0.0	1	0.5	0.431	0.00	n.a.	0.481	0.00	n.a.
B*13	3.2	4.7	8	3.3	11	5.6	10	5.5	0.277	0.59	0.23-1.53	0.960	1.02	0.42-2.47
B*14	2.5	1.6	2	0.8	5	2.6	3	1.6	0.656	0.50	0.08-3.03	0.725	1.56	0.37-6.63
B*15	8.0	8.7	28	11.7	13	6.6	13	7.1	0.120	1.72	0.86-3.42	0.845	0.92	0.42-2.05
B*18	4.6	4.9	9	3.8	14	7.1	7	3.8	0.959	0.97	0.36-2.67	0.162	1.92	0.76-4.88
B*27	4.3	4.7	8	3.3	5	2.5	16	8.8	**0.030**	**0.36**	**0.15-0.86**	**0.010**	**0.27**	**0.10-0.76**
B*35	10.0	11.3	24	10.0	25	12.8	21	11.5	0.612	0.85	0.46-1.58	0.718	1.12	0.6-2.08
B*37	1.5	0.6	1	0.4	2	1.0	1	0.5	1.000	0.76	0.05-12.19	1.000	1.87	0.17-20.76
B*38	2.2	1.6	3	1.3	5	2.6	2	1.1	1.000	1.14	0.19-6.89	0.451	2.36	0.45-12.3
B*39	2.0	2.8	5	2.1	6	3.1	6	3.3	0.438	0.62	0.19-2.08	0.896	0.93	0.29-2.93
B*40	6.6	7.9	19	7.9	16	8.2	14	7.7	0.932	1.03	0.5-2.12	0.866	1.07	0.51-2.25
B*41	0.9	1.9	2	0.8	4	2.0	6	3.3	0.081	0.25	0.05-1.24	0.531	0.61	0.17-2.2
B*44	12.8	10.5	25	10.4	17	8.7	23	12.6	0.477	0.80	0.44-1.47	0.211	0.66	0.34-1.27
B*45	0.6	0.2	1	0.4	0	0.0	1	0.0	1.000	0.76	0.05-12.19	1.000	0.00	n.a.
B*47	0.4	0.2	1	0.4	0	0.0	1	0.0	1.000	0.76	0.05-12.19	1.000	0.00	n.a.
B*48	0.1	0.2	0	0.0	0	0.0	1	0.5	0.431	0.00	n.a.	0.481	0.00	n.a.
B*49	1.4	0.5	1	0.4	0	0.0	2	1.1	0.580	0.38	0.03-4.19	0.231	0.00	n.a.
B*50	1.3	0.6	2	0.8	0	0.0	2	1.1	1.000	0.76	0.11-5.42	0.231	0.00	n.a.
B*51	6.2	7.1	16	6.7	17	8.7	11	6.0	0.796	1.11	0.5-2.45	0.329	1.48	0.67-3.24
B*52	0.9	1.1	3	1.3	1	0.5	3	1.6	1.000	0.76	0.15-3.79	0.355	0.31	0.03-2.97
B*55	1.4	1.8	6	2.5	4	2.0	1	0.5	0.247	4.64	0.55-38.89	0.373	3.77	0.42-34.06
B*56	0.9	0.3	2	0.8	0	0.0	0	0.0	0.508	n.a.	n.a.	1.000	n.a.	n.a.
B*57	3.6	3.4	8	3.3	6	3.1	7	3.8	0.778	0.86	0.31-2.42	0.676	0.79	0.26-2.39
B*58	1.1	0.6	0	0.0	4	2.0	0	0.0	1.000	n.a.	n.a.	0.124	n.a.	n.a.

[+] The HLA class I allele frequencies were obtained from allelefrequencies.net and include 11407 individuals from the German bone marrow registry; *p-values were calculated using either χ^2 or Fisher's exact test depending on group size, significant values are marked in red; OR: odds ratio; CI: confidence interval; n.a.: not applicable

The distributions of HLA-alleles between the general German population and the PWID cohort were very similar and no significant differences could be found (table 4.2). A comparison of the HLA-allele frequencies within the PWID cohort between chronically GT1 or GT3 infected patients and patients with resolved infection only showed significant differences for three HLA class I alleles.

The frequency of HLA-A*29 was decreased in patients with GT3 infection compared to patients with resolved infection (0.0% vs. 2.7%, p=0.025). HLA-B*08 was significantly more common in GT1 infected patients than in patients with resolved infection (12.5% vs. 6.0%, p=0.027), while HLA-B*27 was significantly enriched in patients with resolved infection (8.8%) compared to both GT1 (3.8%) and GT3 (2.0%) infected patients (p=0.030 and p=0.010, respectively).

In order to avoid false positive results due to either too small group sizes or too many simultaneous comparisons, multiple logistic regression analysis was performed in cooperation with the Department of Bioinformatics, University of Duisburg-Essen, Essen (table 4.3). The increased frequency of HLA-B*08 in GT1 infected patients compared to patients with resolved infection could be confirmed in this analysis (p=0.037). HLA-B*27 was also still significantly more common in patients with resolved infection compared to patients with GT3 infection (p=0.008), while this allele was only borderline enriched compared to GT1 (p=0.063) according to this analysis. The increased frequency of HLA-A*29 in resolved patients compared to GT3 infected patients was calculated as non-significant (p=1.000), indicating a false positive result during the first analysis.

Table 4.3: Multiple logistic regression analysis of HLA class I alleles associated with viral clearance

	GT1 vs. RNA neg			GT3 vs RNA neg		
HLA-allele	p-value	OR	95% CI	p-value	OR	95% CI
A*29				1.000	0.00	
B*08	0.037	2.22	1.08-4.56			
B*27	0.063	0.4	0.17-0.94	0.008	0.22	0.07-0.66

Overall, these findings seem to indicate that HLA-B*08 has a detrimental effect during HCV GT1 infection, whereas HLA-B*27 seems to be protective against both GT1 and GT3 infection. The protective effect during GT3 infection was unexpected, as the known immunodominant HLA-B*27-restricted epitope is only present in GT1 (Neumann-Haefelin et al., 2006, 2010).

4.2.3 Influence of genetic host factors on the HCV-specific CD8$^+$ T cell response

As we could show that some genetic host factors like the *IFNL3* rs12979860 C/C genotype and HLA-B*27 are correlated with spontaneous clearance of HCV infection in our PWID cohort, we next tried to elucidate the mechanisms behind theses associations in more detail.

While the protective effect of certain HLA class I alleles is almost certainly associated with specific CD8$^+$ T cell responses against HCV, there also might be a link to CD8$^+$ T cell immunity in the case of the *IFNL3* genotype. The C/C genotype has been associated with more symptomatic hepatitis and jaundice and thereby increased cytotoxic activity in the liver that is likely to be caused by cytotoxic CD8$^+$ T cells (Beinhardt et al., 2012; Tillmann et al., 2010). For HLA-B*27 it has been shown that the protective effect is associated with the immunodominant CD8$^+$ T cell epitope NS5B$_{2841}$ ARMILMTHF (Neumann-Haefelin et al., 2010). We therefore wanted to see whether the *IFNL3* genotype and the HLA-B*27 status of the patient has any influence on the CD8$^+$ T cell response.

For this purpose, magnitude and breadth of the CD8$^+$ T cell response in 115 PWID was determined via IFN-γ staining after expansion and restimulation with previously described optimal peptides (see table 2.5). For each patient only the peptides matching the patient's HLA-type were tested.

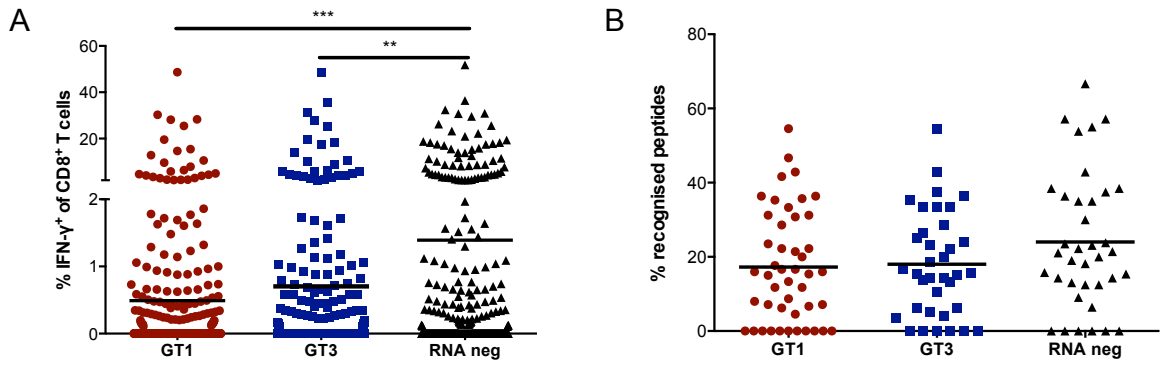

Figure 4.3: Magnitude and breadth of the CD8$^+$ T cell response according to infection outcome

A The relative frequency of CD8$^+$IFN-γ$^+$ T cells after 10 days of *in vitro* expansion in the presence of HLA-matched peptides is shown. Each symbol represents the CD8$^+$ T cell response to one peptide. **B** The percentage of reactive peptides of all tested HLA-matched peptides is shown for each individual. Horizontal lines indicate the mean. p-values were calculated using the Kruskal-Wallis test (**: p<0.01, ***: p<0.001).

The magnitude of the $CD8^+$ T cell response as measured by the percentage of $CD8^+IFN\text{-}\gamma^+$ T cells directed against each peptide was significantly increased in patients with resolved HCV infection compared to patients with either GT1 or GT3 infection (figure 4.3A). Additionally, the percentage of reactive peptides of all tested HLA-matched peptides for each individual was used as an indicator of the breadth of the $CD8^+$ T cell response. Although there was also a trend towards a greater breadth of the $CD8^+$ T cell response in PWID with spontaneously resolved HCV infection, this trend was not statistically significant (figure 4.3B). Interestingly, the breadth and magnitude of the $CD8^+$ T cell response was nearly identical in the two genotypes even though the peptides used for detection were biased toward the GT1 sequence.
To test whether the $CD8^+$ T cell response was influenced by the *IFNL3* genotype, the data on the magnitude and breadth of the $CD8^+$ T cell response was stratified for the *IFNL3* genotype. As can be seen in figure 4.4, no significant differences in the $CD8^+$ T cell response depending on the *IFNL3* genotype could be detected in the different subgroups. There was also no difference in the $CD8^+$ T cell response depending on the *IFNL3* genotype in the total cohort (C/C vs. non C/C genotype; data not shown), suggesting that the protective effect mediated by the *IFNL3* C/C genotype is independent of the $CD8^+$ T cell response.

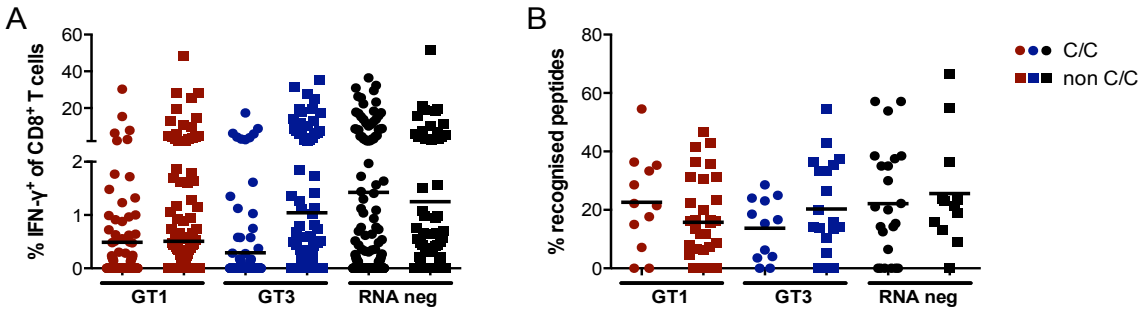

Figure 4.4: Magnitude and strength of the $CD8^+$ T cell response according to the *IFNL3* genotype

The $CD8^+$ T cell response in different subgroups according to the rs12979860 genotype is shown. **A** The relative frequency of $IFN\text{-}\gamma^+CD8^+$ T cells after 10 days *in vitro* expansion for each tested peptide is shown. **B** The percentage of reactive peptides of all tested HLA-matched peptides for each individual is shown. Horizontal lines indicate the mean. p-values were calculated using the Kruskal-Wallis test.

We also divided the two chronic groups and the patients with spontaneously resolved HCV infection into HLA-B*27 positive and negative patients. As can be seen in figure 4.5 the magnitude and breadth of the $CD8^+$ T cell response was slightly elevated in

HLA-B*27 positive patients in both the chronically GT1 infected and resolved patients compared to HLA-B*27 negative patients, but this increase only reached statistically significant levels for the magnitude of the response in GT1 infected patients.

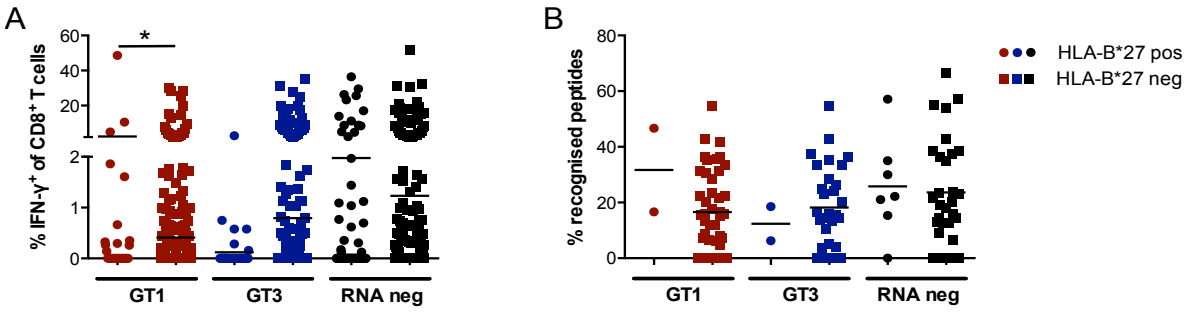

Figure 4.5: Magnitude and breadth of the CD8$^+$ T cell response according to the HLA-B*27 status

The CD8$^+$ T cell response in different subgroups according to the HLA-B*27 status is shown. **A** The relative frequency of IFN-γ$^+$CD8$^+$ T cells after 10 days *in vitro* expansion for each tested peptide is shown. **B** The percentage of reactive peptides of all tested HLA-matched peptides for each individual is shown. Horizontal lines indicate the mean. p-values were calculated using the Kruskal-Wallis test (*: $p<0.05$).

For GT3 infected patients the opposite effect could be observed: There was a slight decrease in both the breadth and the magnitude of the overall CD8$^+$ T cell response, but the difference was not statistically significant.

Overall, our data for the strength of the CD8$^+$ T cell response is probably not conclusive, as too few chronically infected patients were included in this study (2 per group) and the majority of tested peptides were skewed towards a GT1 background. In addition, only 4 HLA-B*27 epitopes were tested, one of which was restricted by the HLA-B*27:02 subtype known to be less common in the general German population than the HLA-B*27:05 subtype.

We therefore wanted to see whether we could identify novel HLA-B*27-restricted epitopes in GT3 that might mediate the protective effect of the HLA-B*27 genotype.

4.3 Identification of new HLA-B*27-restricted epitopes in GT3a

In order to identify novel HLA-B*27-restricted epitopes in GT1a, GT1b and GT3a, the consensus sequences of all three genotypes were entered into the MHC-I Binding Prediction Tool. The 9mers with the highest prediction scores for binding to HLA-B*27:05, which is the most common HLA-B*27 allele with a frequency of 3.4% in the general German population, were synthesized (see table 2.6) and further analysed. Additionally, the known HLA-B*27-restricted epitopes $p7_{780}$ GRWVPGAAY (GT1a), $NS3_{1492}$ GRGKPGIYRF (GT1a), GRGRLGTYRY (GT3a) and its GT1b variant GRGRRGIYRF (GT1b), $NS5B_{2841}$ ARMILMTHF (GT1a/1b) and its GT3a variant $NS5B_{2841}$ VRMVMMTHF (GT3a) and $NS5B_{2936}$ GRAAICGKY (GT1a) were included in all further experiments (Giugliano et al., 2009; Neumann-Haefelin et al., 2006). The known epitope $NS5B_{2820}$ ARHTPVNSW (GT1a/1b/3a) was excluded because is it known to bind to HLA-B*27:02 instead of HLA-B*27:05 (Nitschke et al., 2014). HLA-B*27:02 is rare in the general German population.

4.3.1 Identification of two novel vigorously targeted HLA-B*27-restricted epitopes against GT3a

As a first step, the known and predicted HLA-B*27-restricted peptides were tested in an *in vitro* assay to determine whether $CD8^+$ T cell responses against these epitopes could be detected in HLA-B*27-positive PWID. For this purpose PBMCs from 13 PWID were expanded for 10 days in the presence of these peptides, before the IFN-γ production by $CD8^+$ T cells was detected via ICS (figure 4.6).

As shown in figure 4.6A (marked with blue rectangles), IFN-γ-producing $CD8^+$ T cells against three novel HLA-B*27-restricted epitopes in GT3a could be detected in different PWID. One of the tested PWID displayed a response against the $NS5A_{2094}$ epitope, while two PWID showed a $CD8^+$ T cell response against the $NS2_{840}$ epitope. A response against the $NS2_{942}$ epitope could be detected in 5 PWID, while 3 PWID had a detectable response against the known immunodominant GT1 $NS5B_{2841}$ epitope.

The magnitude of the response per epitope was highest for the known immunodominant $NS5B_{2841}$ epitope (mean: 4.82%), followed by the two new GT3a epitopes NS_{840} and $NS2_{942}$ (means 2.94% and 2.67%, respectively; figure 4.6B).

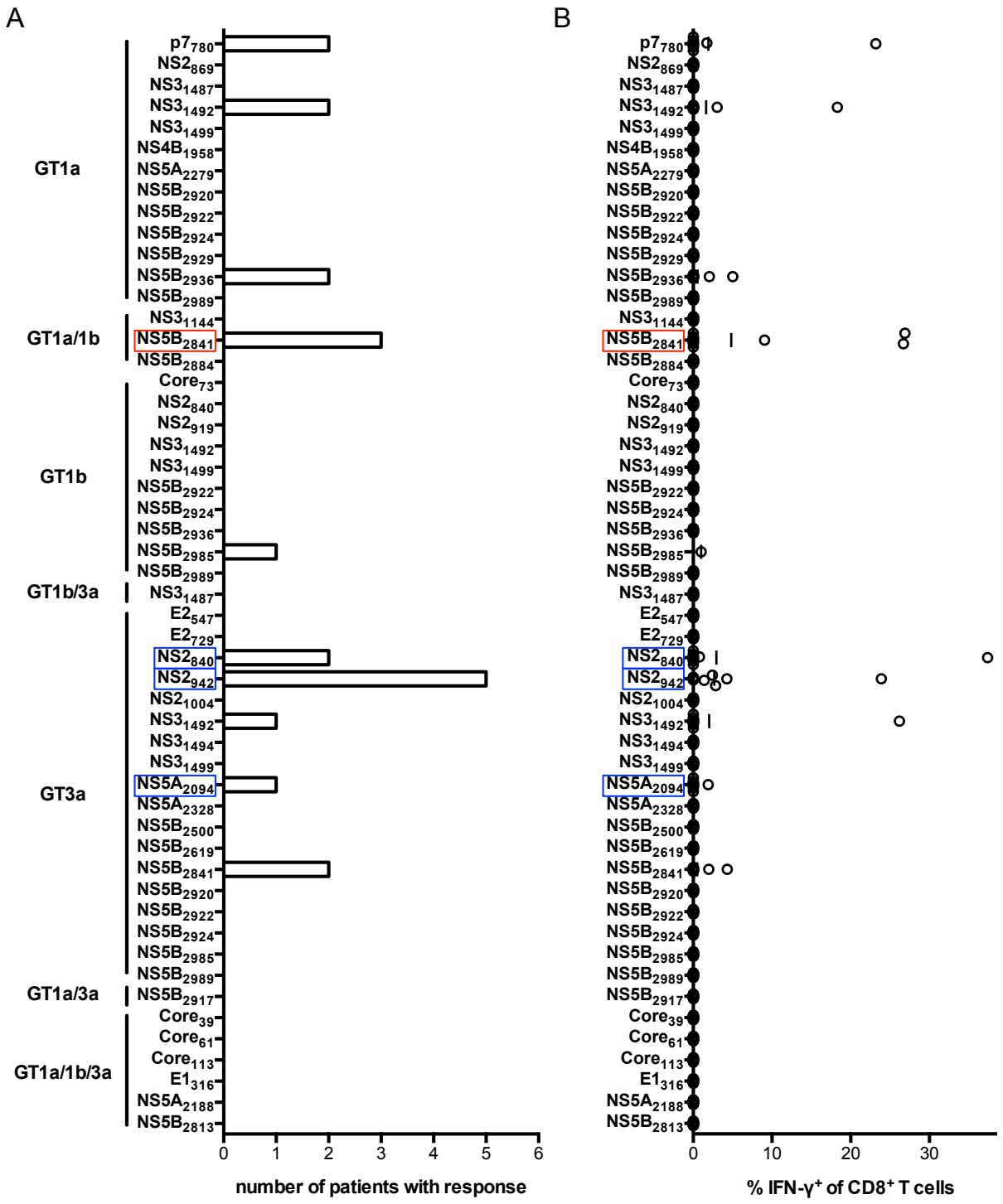

Figure 4.6: CD8$^+$ T cell response against HLA-B*27-restricted epitopes

The relative frequency of CD8$^+$IFN-γ$^+$ T cells after 10 days of *in vitro* expansion in the presence of predicted and already known HLA-B*27-restricted peptides was examined in 13 HLA-B*27 positive, HCV seropositive PWID via ICS and flow cytometric analysis. **A** Recognition frequencies are shown for each tested peptide. **B** Magnitude of the CD8$^+$ T cell response is shown for each peptide. Each circle represents the CD8$^+$ T cell response of one patient. A line indicates the mean. The known immunodominant GT1 epitope is marked with a red rectangle, three novel GT3 epitopes are marked with blue rectangles.

In addition, $CD8^+$ T cell responses could also be detected against the other known epitopes $p7_{780}$ (GT1a), $NS3_{1492}$ (GT1a), $NS3_{1492}$ (GT3a), $NS5B_{2841}$ (GT3a) and $NS5B_{2936}$ (GT1a) in one or two patients each. A weak response against the previously unknown GT1b epitope $NS5B_{2985}$ was also detected in one patient.
Overall, the two new GT3a-specific epitopes located in NS2 seemed to be comparable to the known GT1-specific $NS5B_{2841}$ epitope in both the number as well as the magnitude of the responses and were therefore analysed further.

4.3.2 Peptide binding to HLA-B*27:05 is high for epitopes that elicit a $CD8^+$ T cell response

As a second step the relative peptide binding of the predicted and known HLA-B*27 epitopes to HLA-B*27:05 was tested in UV-induced peptide-exchange reactions using a high affinity-binding peptide as a control to be able to compare experimental results with those obtained by *in silico* prediction (figure 4.7).

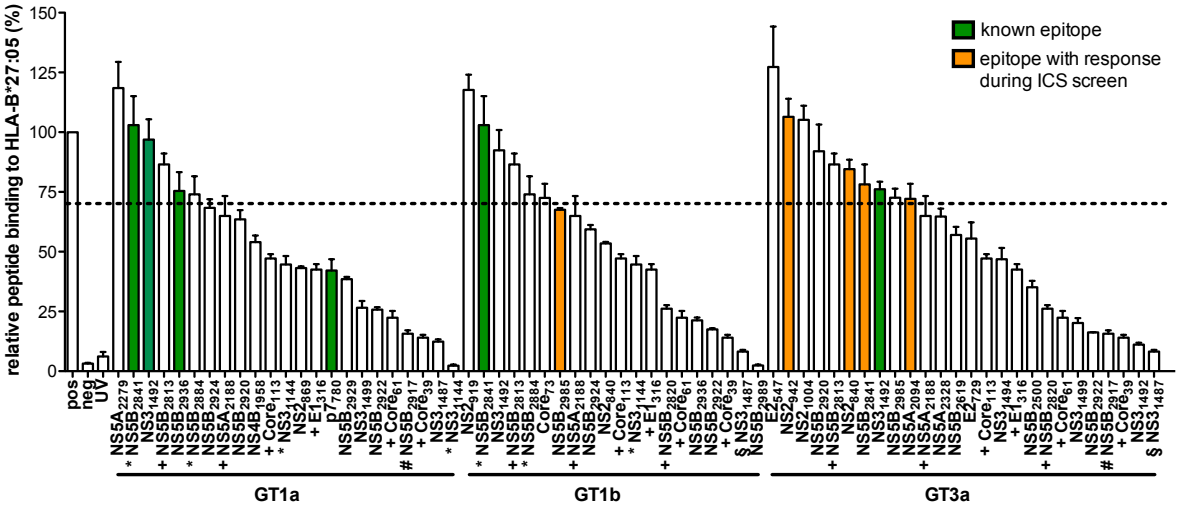

Figure 4.7: Peptide binding to HLA-B*27:05

Binding of peptides containing predicted HLA-B*27:05 epitopes was tested using UV-induced peptide-exchange reactions. The experiment was performed twice and the bars indicate the mean of the percentage of binding relative to a high affinity control (Toebes et al., 2006). *: sequence identical between GT1a and GT1b, +: sequence identical between GT1a, GT1b and GT3a, #: sequence identical between GT1a and GT3a, §: sequence identical between GT1b and GT3a.

Except for the known peptides mentioned above, only the following 12 HLA-B*27 restricted epitopes reached binding affinities above 70% of the positive control: $Core_{73}$ (GT1b), $E2_{547}$ (GT3a), $NS2_{840}$ (GT3a), $NS2_{919}$ (GT1b), $NS2_{942}$ (GT3a), $NS2_{1004}$ (GT3a), $NS5A_{2094}$ (GT3a), $NS5A_{2279}$ (GT1a), $NS5B_{2813}$ (GT1a/1b/3a),

$NS5B_{2884}$ (GT1a/1b) and $NS5B_{2920}$ (GT3a) (figure 4.7). Nearly all epitopes that had a detectable response during the ICS screen (marked in orange in figure 4.7) had affinities above 70% of the positive control. This was not unexpected as a high binding affinity to the MHC molecule is a necessary but not sufficient criterion for an epitope that elicits a $CD8^+$ T cell response. The only exceptions were the GT1b epitope $NS5B_{2985}$, which had a mean binding affinity of 67.6% and was thereby only slightly below the 70% cut-off, and the GT1a $p7_{780}$ epitope, which had a mean binding affinity of 42.2%.

Surprisingly, more than 50% (28 of 54) of the tested peptides had a binding affinity below 50% of the positive control even though they were indicated as strong binders during the initial prediction step. Interestingly, the GT3a epitopes $NS2_{840}$ and the $NS2_{942}$, which were the most promising candidates for novel immunodominant GT3a epitopes in the ICS screen, also had the highest binding affinities of the GT3a epitopes that elicited a $CD8^+$ T cell response.

4.3.3 *Ex vivo* frequency of HLA-B*27-restricted $CD8^+$ T cells is comparable for the GT3a-specific $NS2_{940}$ and the GT1-specific $NS5B_{2841}$ epitopes

As our initial analysis of the $CD8^+$ T cell response against HLA-B*27 restricted epitopes consisted of an ICS screen and was therefore biased towards HCV-specific cells that could be expanded and were functional, i.e. produced IFN-γ, we wanted to examine the $CD8^+$ T cell responses directed against the two GT3a epitopes in NS2 directly *ex vivo*. As a comparison the immunodominant GT1 epitope $NS5B_{2841}$ was also included in this analysis.

For this purpose we used MHC class I dextramers, which consist of a dextran polymer backbone coupled to several MHC-peptide complexes and fluorochromes. The dextramers can thus bind to several TCRs on the surface of a $CD8^+$ T cell and allow direct staining and sensitive detection of HCV-specific $CD8^+$ T cells including functionally impaired and exhausted $CD8^+$ T cells.

25 HLA-B*27 positive PWID were stained with MHC class I dextramers and the frequency of $NS5B_{2841}$-, $NS2_{840}$- and the $NS2_{942}$-specific $CD8^+$ T cells was determined via flow cytometry. In 18 PWID $CD8^+$ T cells directed against the previously described $NS5B_{2841}$ epitope were detectable and in 13 PWID $CD8^+$ T cells directed against the $NS2_{942}$ epitope were detectable (figure 4.8A). $CD8^+$ T cell directed against the $NS2_{840}$ epitope were detectable in fewer PWID (n=6). The mean

magnitude of the dextramer response was in a similar range for the $NS5B_{2841}$ epitope (0.130%) and the $NS2_{942}$ epitope (0.116%), while the response against the $NS2_{840}$ epitope was again lower (0.054%) (figure 4.8B).

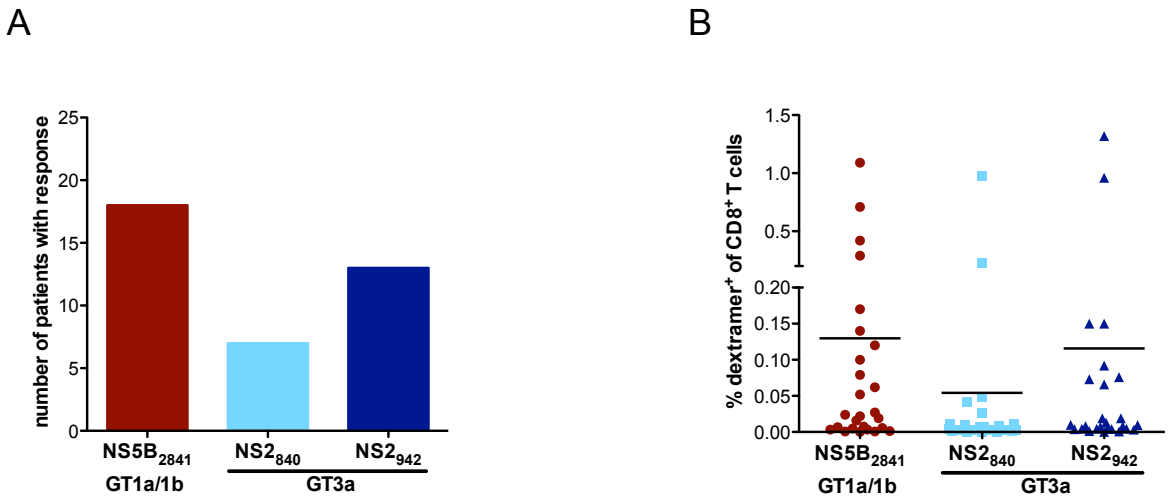

Figure 4.8: *Ex vivo* CD8$^+$ T cell response against HLA-B*27 restricted epitopes

The relative frequency of HCV-specific CD8$^+$ T cells was determined via MHC class I dextramer staining directly after thawing. **A** Recognition frequencies are shown for each tested dextramer. Dextramer responses were counted as positive if they constituted at least 0.004% of the total CD8$^+$ T cell population and a distinct population could be identified. **B** Magnitude of the CD8$^+$ T cell response is shown for each epitope. Each circle represents the CD8$^+$ T cell response of one patient. A line indicates the mean.

4.3.4 Memory phenotype of HLA-B*27 restricted CD8$^+$ T cells

To further characterise the CD8$^+$ T cell response directed against the two new NS2 epitopes as well as the $NS5B_{2841}$ epitope, we wanted to take a closer look at the memory phenotype of the CD8$^+$ T cell responses. The IL-7 receptor α-chain, also known as CD127, is a key molecule for the maintenance of memory T cell populations and has been previously associated with superior functionality against HCV (Seigel et al., 2013). Moreover, upregulation of CD127 on HCV-specific CD8$^+$ T cells was observed after either viral clearance or upon viral sequence variation (Kasprowicz et al., 2010). We therefore wanted to utilise this marker and correlate it to the proliferation potential of the HCV-specific CD8$^+$ T cells.

To achieve this, specific cells were stained with MHC class I dextramers directly after thawing (*ex vivo*) and after 7 days of peptide-specific expansion (day 7) and the CD127 expression of CD8$^+$dextramer$^+$ cells was determined *ex vivo* (figure 4.9A). The fold increase of CD8$^+$dextramer$^+$ cells between the two time points was then

calculated and correlated to either high or low CD127 expression on these cells (figure 4.9B).

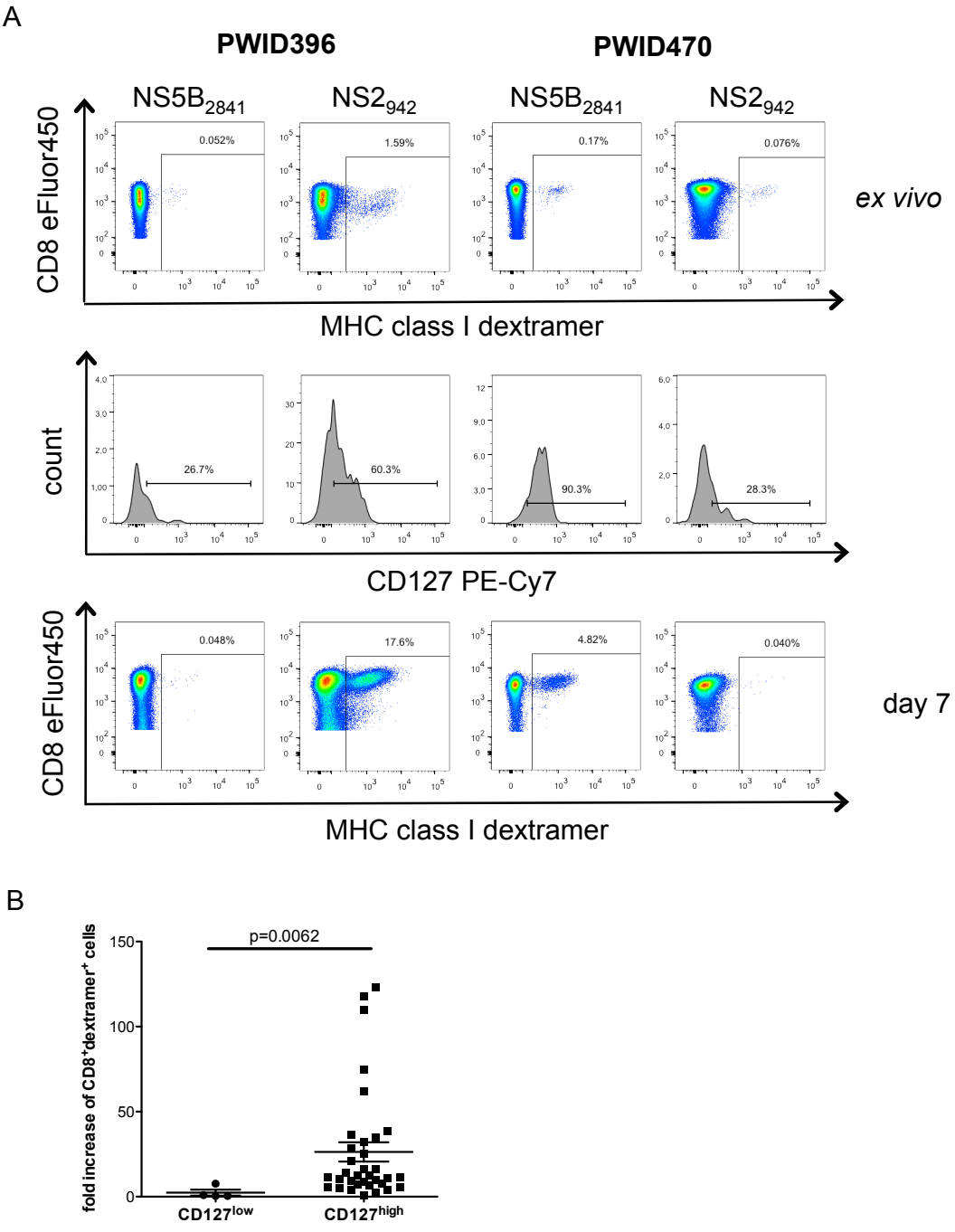

Figure 4.9: Memory phenotype of HLA-B*27 restricted HCV-specific CD8$^+$ T cells

A Frequencies of NS5B$_{2841}$-, NS2$_{840}$- and the NS2$_{942}$-specific CD8$^+$ T cells were determined directly after thawing and after 7 days of peptide-specific expansion via MHC class I dextramer staining and subsequent flow cytometric analysis. CD127 expression on HCV-specific CD8$^+$ T cells was determined *ex vivo*. Exemplary FACS plots are shown for two PWID with responses against the NS5B$_{2841}$ and the NS2$_{942}$ epitope. **B** Fold increase of CD8$^+$dextramer$^+$ T cells between day 0 and day 7 was calculated and stratified for CD127 expression *ex vivo*. Cells were classified as CD127^{low}, if the CD127 expression was below 60%, otherwise cells were classified as CD127^{high}. The p-value was calculated using Mann Whitney test.

The fold increase of $CD8^+$dextramer$^+$ cells between day 0 and day 7 with low CD127 expression (under 60%) was significantly lower compared to that of $CD8^+$dextramer$^+$ cells with high CD127 expression. This is in line with previous data that indicates that HCV-specific cells with low CD127 expression have a lower proliferative potential than those with high CD127 expression (Bengsch et al., 2010). In this study by Bengsch *et al.* high CD127 expression on HCV-specific cells of chronic patients was also linked to viral escape in the respective epitope. The two lowest CD127 expression values were found in PWID396 and PWID470 (figure 4.9A), who were both chronically HCV infected. Consequently, we wanted to examine the viral sequence in these epitopes in more detail.

4.3.5 Low CD127 expression is present on HCV-specific cells that target epitopes with intact viral sequences in chronic PWID

First, we examined the viral sequence in the $NS5B_{2841}$ epitope in GT1 infected patients (table 4.4). The majority of patients had two or more sequence polymorphisms, while two patients had none or only one substitution. The pattern of sequence polymorphisms was similar to that observed in other studies of this epitope (Dazert et al., 2009; Neumann-Haefelin et al., 2006, 2010). In PWID396, who had low CD127 expression on $CD8^+$ T cells targeting the $NS5B_{2841}$ epitope (figure 4.9A), the epitope had an alanine to valine substitution in the first position of the epitope. Even though the viral sequence was not completely intact, it has been shown that this viral polymorphism is still completely cross-reactive with T cells targeting the original epitope. This substitution on its own does therefore not confer viral escape (Dazert et al., 2009). The other patient without substitutions in the $NS5B_{2841}$ epitope was PWID545, for whom no responses against this epitope could be detected.

Table 4.4: Autologous viral sequence of the $NS5B_{2841}$ epitope in GT1 infected patients

ID	GT	$NS5B_{2841}$								
consensus	1a/1b	A	R	M	I	L	M	T	H	F
PWID093	1a	.	.	.	V	M	.	.	.	.
PWID159	1a	V	.	.	V	.	L	.	.	.
PWID221	1a	V	.	.	V	.	.	.	.	.
PWID309	1a	V	.	.	.	.	L	.	.	.
PWID425	1a	V	.	.	.	.	L	.	.	.
PWID545	1a	.	.	.	.	.	.	.	.	.
PWID396	1b	V	.	.	.	.	.	.	.	.

Next, we also sequenced the two NS2 epitopes in GT3a infected patients (table 4.5).

Table 4.5: Autologous viral sequence of the $NS2_{840}$ and $NS2_{942}$ epitopes in GT3a infected patients

HLA-B*27	ID	GT	$NS2_{840}$									p-value[+]	$NS2_{942}$									p-value
	consensus	3a	G	R	L	I	W	W	N	Q	Y		G	R	W	F	N	T	Y	L	Y	
pos	PWID319	3a	S	.	.	.	.	.	.	.	.	0.2047	.	.	.	.	.	.	.	.	.	0.0016
	PWID390		S	.	.	.	.	.	.	.	.		.	.	.	C	.	.	.	.	.	
	PWID429		.	.	.	V	.	.	.	R	.		A	.	.	.	.	.	.	.	.	
	PWID470		S	.	I	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID524		A	.	.	M	.	.	.	.	.		.	K	.	.	.	.	.	.	.	
	FB001		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	FB002		S	.	.	.	.	.	.	.	.		.	.	.	C	.	.	.	.	.	
	FB003		.	.	.	M	.	.	.	.	.		.	.	Y	.	.	.	.	.	.	
	FB004		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	FB007		.	.	I	M	.	.	.	.	.		.	K	.	.	.	.	.	.	.	
	FB008		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	FB010		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	FB011		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	FB012		.	.	.	M	.	.	.	.	.		.	.	.	C	.	.	.	.	.	
	FB013		S	.	.	M	.	.	.	.	.		.	.	.	C	.	.	.	.	.	
	FB014		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
neg	PWID004	3a	S	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID022		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID032		S	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID036		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID044		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID063		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID064		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID070		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID076		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID079		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID092		.	.	.	.	.	.	.	.	.		.	.	L	.	.	.	.	.	.	
	PWID096		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID112		S	.	I	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID113		S	.	I	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID114		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID120		S	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID122		C	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID127		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID132		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID144		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID150		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID163		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID174		.	.	.	M	.	.	.	.	.		.	.	.	C	.	.	.	.	.	
	PWID176		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID182		.	.	.	M	.	.	.	.	.		.	.	F	.	.	.	.	.	.	
	PWID195		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID207		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID217		S	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID240		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID245		S	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID249		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID274		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID281		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID285		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID292		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID295		S	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID300		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID303		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID316		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID331		S	.	.	.	.	.	.	.	.		.	.	.	C	.	.	.	.	.	
	PWID335		S	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID336		S	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID340		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID346		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID347		S	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID350		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID352		.	.	.	M	.	.	.	.	.		.	.	.	L	.	.	.	.	.	
	PWID359		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID382		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID387		.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID449		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID463		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID467		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID468		.	.	.	M	.	.	.	.	.		.	.	.	C	.	.	.	.	.	
	PWID501		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
	PWID503		.	.	.	M	.	.	.	.	.		.	.	.	.	.	.	.	.	.	

Positions with sequence polymorphisms are shaded in gray; [+]: p-values for the association between the frequency of sequence polymorphisms and the presence of HLA-B*27 were calculated using Fisher's exact test.

The second chronically infected patient with low CD127 expression on its HCV-specific $CD8^+$ T cells (PWID470, $NS2_{942}$-specific $CD8^+$ T cells) displayed no mutations in this epitope. As in the study by Bengsch *et al.* HCV-specific $CD8^+$ T cells with very low CD127 expression had low proliferative capacity probably caused by ongoing antigen triggering due to lack of escape mutations in the corresponding epitopes. The second patients without substitutions in the $NS2_{942}$ epitope (PWID319) again had no detectable $CD8^+$ T cell response against this epitope.

4.3.6 Escape mutations in the $NS2_{942}$ epitope

As only very few sequences were available in these two regions for GT3a in the Los Alamos HCV sequence database, we sequenced the autologous virus from 56 chronically 3a infected HLA-B*27-negative PWID (table 4.5). For the HLA-B*27-positive patients we included another 11 HLA-B*27-positive patients chronically infected with GT3a from a previously published cohort of the University of Freiburg (Neumann-Haefelin et al., 2010), because only very few PWID with GT3a infection were present in our cohort. Overall, the sequence variability was greater in the $NS2_{840}$ epitope compared to the $NS2_{942}$ epitope and here position 1 and 4 showed the greatest variability (figure 4.10A). Importantly, in position 4 isoleucine and methionine were both equally likely and no difference in the frequency could be found between HLA-B*27 positive and HLA-B*27 negative PWID indicating that this residue was not under selection pressure by HLA-B*27. Polymorphisms at different positions of the epitope were not more likely to occur in HLA-B*27 positive patients compared to HLA-B*27 negative patients (p=0.2047, table 4.5). Collectively, there was no evidence for HLA-B*27 mediated escape in the $NS2_{840}$ epitope.

The $NS2_{942}$ epitope was more conserved with 89.3% of sequences corresponding to the prototype. The only positions showing any variability were positions 2, 3 and 4 of the epitope (figure 4.10B). When the frequency of sequence polymorphisms in the whole epitope were compared between HLA-B*27 positive and negative patients, polymorphisms in the $NS2_{942}$ epitope were significantly more frequent in HLA-B*27 positive patients (p=0.0016, Fisher's exact test) strongly suggesting that the epitope is under selection pressure in the presence of HLA-B*27.

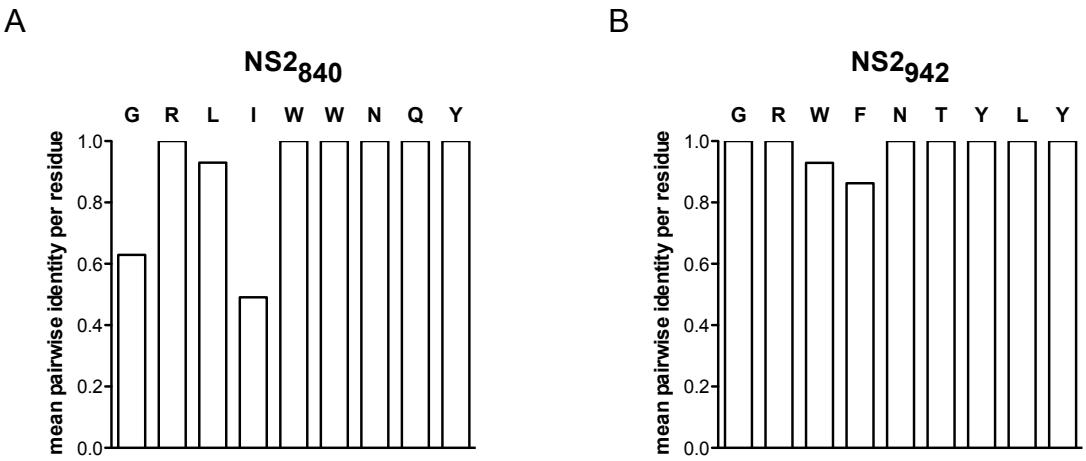

Figure 4.10: Sequence variability of the $NS2_{840}$ and $NS2_{942}$ epitopes in HLA-B*27 negative PWID

Mean pairwise sequence identity per individual residue was determined using Geneious.

To analyse the relevance of these substitutions on the $CD8^+$ T cell response, we performed cross-reactivity studies via ICS. First, we examined the polymorphism between isoleucine and methionine at position 4 of the $NS2_{840}$ epitope in more detail. As can be seen in figure 4.11A, $CD8^+$ T cell responses against the GRLIWWNQY variant (GT3a, dark blue bars) also recognised the GRLMWWNQY variant (GT3a V1, light blue bars). In both cases $CD8^+$ T cells could also be expanded in the presence of the GT3a V1 variant, but the frequency of expanded cells differed considerably between patients from barely detectable in PWID488 to higher frequencies than the prototype GT3a variant in PWID519. The I4M polymorphism seems to have an influence on the $CD8^+$ T cell response, but there is little evidence for immune escape based on the cross-reactivity profile.

Next, we wanted to examine the autologous viral sequences of the HLA-B*27 positive chronically GT3a infected PWID in more detail. As a first step, we tried to expand $CD8^+$ T cell responses with peptides corresponding to the autologous viral sequence of the $NS2_{840}$ and $NS2_{942}$ epitope in patients harbouring the respective sequence. After 10 days of expansion the cells were restimulated with the same peptides, but no response could be detected in any of the patients (data not shown). This result was not surprising, as no response could be detected after expansion and restimulation with the prototype sequences, either (data not shown).

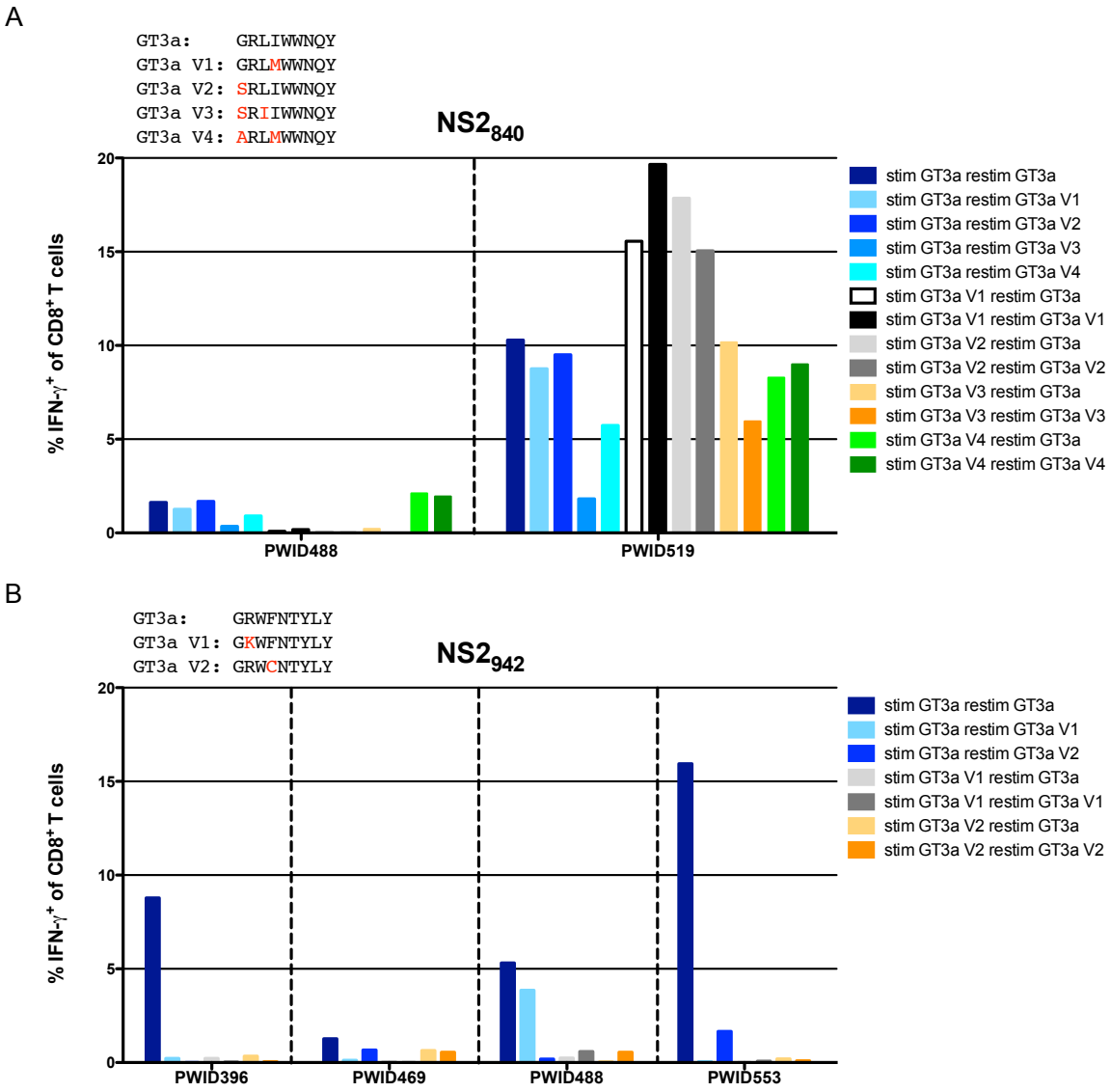

Figure 4.11: CD8$^+$ T cell response against the prototype HLA-B*27-restricted NS2$_{840}$ and NS2$_{942}$ epitopes and the corresponding autologous viral sequences

The relative frequency of CD8$^+$IFN-γ$^+$ T cells after 10 days of *in vitro* expansion in the presence of the HLA-B*27-restricted peptides NS2$_{840}$ (**A**) or NS2$_{942}$ (**B**) was examined in HLA-B*27 positive, PWID who had a measurable response against either of these epitopes during the initial ICS screen. Cross-reactivity of CD8$^+$ T cells was evaluated by ICS for IFN-γ after restimulation with either the GT3a or autologous viral sequence peptide (GT3a V1-4). Each bar represents the CD8$^+$ T cell response to one peptide.

As a second step, expansion with the autologous viral sequence peptides as well as the prototype sequence was performed in PWID that displayed a response against the prototype sequence (figure 4.11).

For the NS2$_{840}$ epitope, the CD8$^+$ T cell line expanded in the presence of the prototype sequence GRLIWWNQY could also elicit a response when it was restimulated with HLA-B*27-associated variant epitope sequences, although the

magnitude of the response differed (figure 4.11A). In both tested patients the magnitude of the $CD8^+$ T cell response was nearly identical between the prototype sequence and the SRLIWWNQY variant (GT3a V2). The magnitude of the response was reduced by nearly 50% for the ARLMWWNQY variant (GT3a V4), while it was even lower for the SRIIWWNQY variant (GT3a V3). The ARLMWWNQY variant could also be used to expand $CD8^+$ T cells in both patients, while for the other two variants this was only possible for PWID519. In all cases the $CD8^+$ T cells expanded in the presence of the autologous viral sequences were fully cross-reactive with the prototype sequence. Collectively, these data suggest that the variant V3 may act as an escape variant, while the other variants are not associated with immune escape. Taken together, most of the sequence variations in the $NS2_{840}$ epitope are immunogenic and unlikely to be the product of immune selection pressure.

In contrast, $CD8^+$ T cells expanded with the $NS2_{942}$ prototype sequence displayed no or minimal cross-reactivity with the GKWFNTYLY variant (GT3a V1) or the GRWCNTYLY variant (GT3a V2) in the four tested patients (figure 4.11B). Only PWID488 showed some cross-reactivity between the prototype sequence and the GKWFNTYLY variant (GT3a V1). This was quite surprising, as the substitution for this variant is at position 2 of the epitope and therefore in one of the anchor positions for MHC binding. Mutations in this position usually result in impaired binding to the MHC molecule and would therefore result in an accelerated off-rate of the variant peptide from the MHC molecule. To exclude that this result was an artefact of the continuous presence of the peptide, we loaded PBMC from another HLA-B*27 positive donor with either the prototype peptide or the autologous viral sequence peptide and washed them extensively before using these cells for restimulation (figure 4.12). This way only peptide properly bound to the MHC molecule of the donor PBMCs was available for restimulation while excess peptide was washed away.

PBMCs pulsed with the prototype peptide elicited $CD8^+$ T cell responses of roughly the same magnitude as those generated by direct peptide stimulation. In contrast, the $CD8^+$ T cell responses caused by PBMCs pulsed with the variant peptide were nearly ten fold reduced compared to those elicited by direct peptide stimulation. In this case the stability of the MHC-peptide complex may not have been sufficient causing the variant peptide to be removed during the washing steps. Responses against the either the prototype or the variant sequences were again barely detectable in the $CD8^+$ T cell line expanded with the variant peptide.

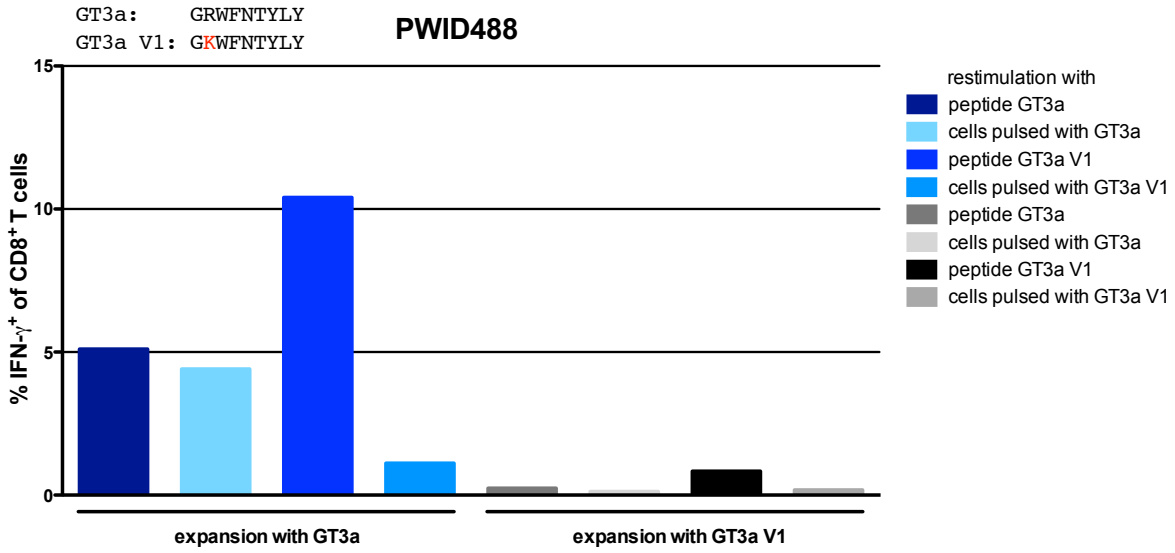

Figure 4.12: CD8$^+$ T cell response against the NS2$_{942}$ epitopes with direct peptide stimulation or peptide loaded cells

The relative frequency of CD8$^+$IFN-γ$^+$ T cells after 14 days of *in vitro* expansion in the presence of the HLA-B*27-restricted peptide NS2$_{942}$ was examined in the HLA-B*27 positive PWID488. Cross-reactivity of CD8$^+$ T cells was evaluated by ICS for IFN-γ after restimulation with either direct peptide stimulation with the GT3a or autologous viral sequence peptide (GT3a V1) or HLA-B*27 positive PBMC pulsed with either peptide.

In summary, there is no evidence for HLA-B*27 mediated escape mutations in the NS2$_{840}$ epitope, as the mutations that occur in this epitope are not more frequent in HLA-B*27 positive patients and do not abrogate recognition by CD8$^+$ T cells recognising the prototype sequence. In contrast, true escape mutations seem to be selected in the NS2$_{942}$ epitope that act either through abrogation of TCR recognition (GRWCNTYLY variant) or through a decrease in binding to the MHC molecule (GKWFNTYLY variant).

4.3.7 Both NS2 epitopes are only immunogenic in GT3a

Next, we wanted to determine, whether these two epitopes might also be immunogenic in GT1a and GT1b or whether cross-reactivity between the different genotypes might occur. As a first step, we looked at the sequences available in the Los Alamos HCV sequence database (table 4.6). Although there was extensive sequence variation in both epitope regions in genotype 1a and 1b, the genotype 3a prototype epitope sequences were absent from all GT1 sequences studied. In fact, all GT1a sequences harboured multiple substitutions (≥ 4 substitutions) and all GT1b

sequences harboured ≥2 substitutions compared to the GT3a prototype sequences in both epitopes.

Table 4.6: Alignment of sequences covering the $NS2_{840}$ and $NS2_{942}$ epitopes from the HCV database

source	GT	$NS2_{840}$	%	$NS2_{942}$	%
tested peptide	3a	GRLIWWNQY		GRWFNTYLY	
HCV sequence database (n=301, n=143)	1a	SWCL..L..	71.10	.ALTG..V.	75.52
		SWCM..L..	4.98	.ALTG..I.	15.38
		SWC...L..	3.32	.ALAG..V.	2.80
		SWCF..L..	3.32	.ALTG..VF	2.10
		SWCL..L.X	2.33	other	4.20
		CWCL..L..	1.66		
		SWCV..L..	1.33		
		.WCL..L..	1.33		
		SWCL..I..	1.33		
		SWCX..L..	1.00		
		other*	16.34		
HCV sequence database (n=250, n=230)	1b	A.....L..	54.80	AALTG..V.	85.65
		AK....L..	23.60	.ALTG..V.	3.91
		A.....S..	4.00	AALTG..I.	2.61
		A..M..L..	2.40	.ALTG..I.	2.17
		AK....S..	2.00	other	4.33
		T.....L..	1.60		
		A.I...L..	1.60		
		A..L..L..	1.20		
		A.F...L..	1.20		
		other	7.6		
HCV sequence database (n=7, n=4)	3a	....	28.57	....	75.00
		...M.....	14.29	ADGS.....	25.00
		A........	14.29		
		S..M.....	14.29		
		A..M.....	14.29		
		...M..X..	14.29		

*: all sequences with a frequency below 1% were summarised as other, at least 2 amino acid differences compared to the GT3a consensus sequence were present

Even though the GT3a prototype sequences differed greatly from both the GT1a and GT1b consensus sequences, we wanted to determine whether the GT1a and GT1b sequences might still elicit $CD8^+$ T cell responses. For this purpose these sequences were also tested in an ICS screen with PBMCs from HLA-B*27-positive PWID, who had either resolved infection or were chronically GT1 infected (figure 4.13A).

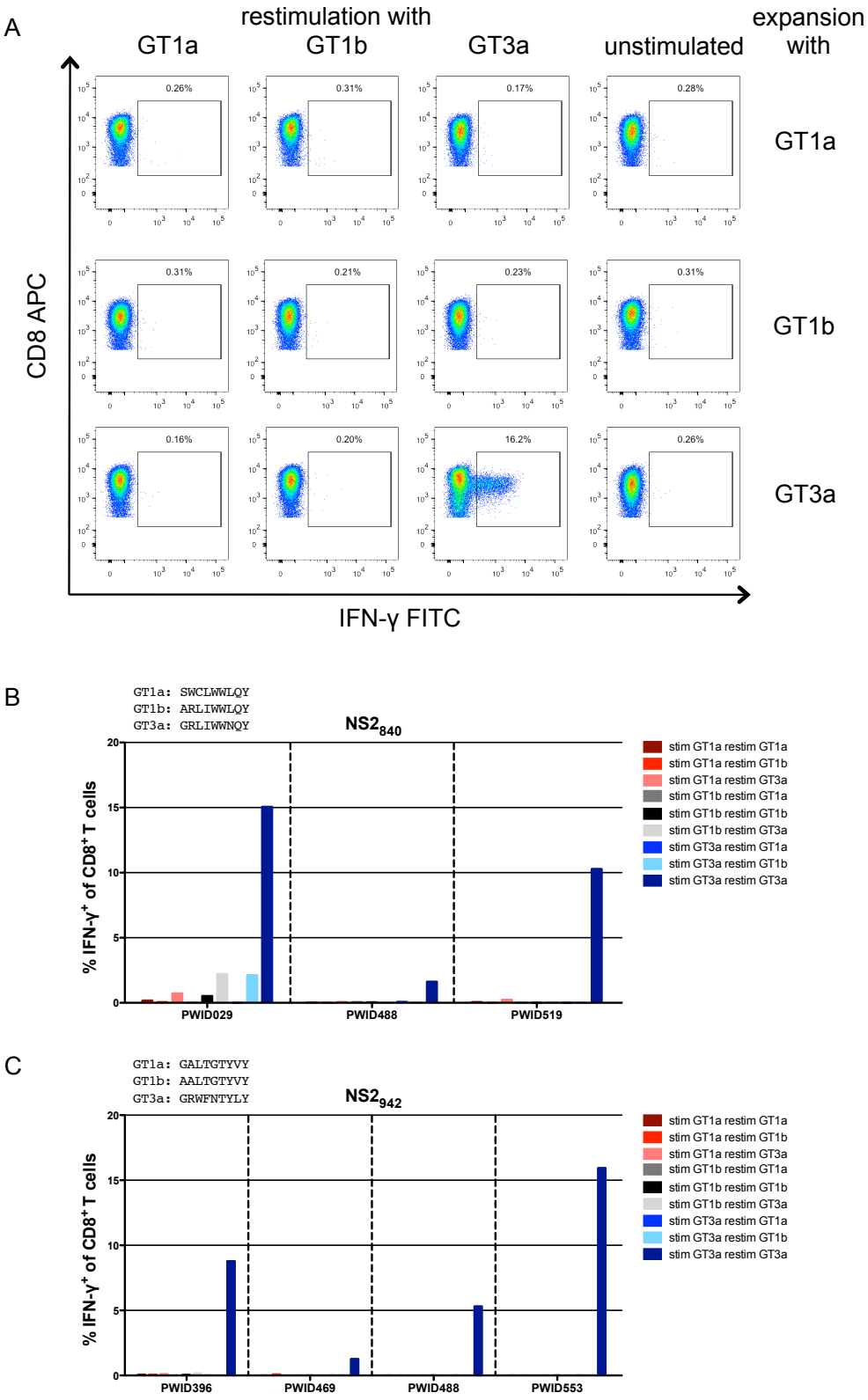

Figure 4.13: CD8$^+$ T cell responses against GT1a, GT1b and GT3a variants of the NS2$_{840}$ and NS2$_{942}$ epitopes

The relative frequency of CD8$^+$IFN-γ$^+$ T cells after 10 days of *in vitro* expansion in the presence of the HLA-B*27-restricted peptides NS2$_{840}$ and NS2$_{942}$ was examined in HLA-B*27 positive PWID via ICS and flow cytometric analysis. **A** Exemplary FACS plots are shown for the NS2$_{942}$ epitope in PWID553. Cross-reactivity of CD8$^+$ T cells was evaluated by ICS for IFN-γ after restimulation with either the GT1a, GT1b or GT3 peptide of the NS2$_{840}$ (**B**) and NS2$_{942}$ (**C**) epitope.

After analysing the $CD8^+$ T cell immune response in 3 PWID, no IFN-γ response was detected after expansion and restimulation with either the GT1a or GT1b peptides, while responses targeting the corresponding GT3a sequence of the $NS2_{840}$ epitope were still readily detectable (figure 4.13B). In one patient (PWID029) there was minimal cross-reactivity between the GT3a variant and the GT1b variant. For the $NS2_{942}$ epitope the $CD8^+$ T cells expanded with the GT3a sequence did not show any cross-reactivity with any of the GT1 sequences. No GT1-specific responses could be detected in any of the four tested patients. Taken together, these data seem to indicate that even though both the $NS2_{840}$ and the $NS2_{942}$ sequences constitute epitopes in GT3a, the corresponding sequences in GT1a and GT1b do not seem to be immunogenic.

Overall, we were able to show that HLA-B*27 is associated with spontaneous clearance of both GT1 and GT3 HCV infection in the PWID cohort. The protective effect of HLA-B*27 during GT1 infection has been previously linked to the immunodominant epitope $NS5B_{2841}$ (Neumann-Haefelin et al., 2006, 2010), which was also the most frequently recognised HLA-B*27 restricted epitope in our cohort. In addition, we could identify two novel HLA-B*27 restricted epitopes in GT3 including one immunodominant epitope associated with mutational escape in HLA-B*27-positive individuals that might explain the protective effect of this HLA molecule during GT3 infection. Importantly, the GT3a-specific $NS2_{942}$ epitope was recognised nearly as often and with a similar magnitude as the GT1-specific $NS5B_{2841}$ epitope.

4.4 T cell responses targeting different variants of the same epitope

Not only genetic host factors, but also genotype-specific differences in the elicited $CD8^+$ T cell response can play an important role in determining the outcome of HCV infection. Our group has previously shown that patients with $CD8^+$ T cells active against both GT1 and GT3 are predominantly found in PWID with resolved infection (Giugliano et al., 2009). These included $CD8^+$ T cell responses that were cross-reactive between the different genotypes as well as genotype-specific responses, in which case two distinct T cell responses targeting the different genotypes could be detected within one patient. Here, we aimed to determine the frequency of co-existing HCV genotype-specific $CD8^+$ T cell populations in highly exposed PWID. We were especially interested in $CD8^+$ T cell responses restricted by HLA molecules that have been shown to be protective during viral infections. These include HLA-B*13 and HLA-B*15, which have been associated with decreased viral loads during human immunodeficiency virus (HIV) infection with clade C (Honeyborne et al., 2007; Tang et al., 2002) and clade B (Frahm et al., 2006), respectively, as well as HLA-B*27 and HLA-B*57, which have been associated with slower disease progression during HIV infection (Goulder and Watkins, 2008; Kaslow et al., 1996; McNeil et al., 1996; Trachtenberg et al., 2003) and spontaneous viral clearance during HCV infection (Kim et al., 2011; Kuniholm et al., 2010; McKiernan et al., 2004; Thio et al., 2002). Only immunodominant epitopes, in which binding to the MHC molecule was not potentially impaired due to substitutions in MHC binding anchor positions, were included in this study.

4.4.1 HLA-B*27 restricted epitopes $NS3_{1492}$ and $NS5B_{2841}$

As a first example we wanted to examine the HLA-B*27 restricted epitope $NS3_{1492}$, because $CD8^+$ T cell responses against both the GT1a and the GT3a variants of this epitope have been described (Giugliano et al., 2009; Neumann-Haefelin et al., 2006), but cross-reactivity and simultaneous occurrence in one patient had not been previously explored. We tested the cross-reactivity of all three variants (GT1a, GT1b, GT3a) in 25 HLA-B*27 positive PWID via ICS staining and flow cytometric analysis. As can be seen in figure 4.14, five PWID displayed a response against at least one variant of this epitope. In two patients a response against both the GT1a and the GT3a sequence was detectable, but no cross-reactivity occurred between these two

variants. Additionally, two patients only had a response against the GT3a sequence, while one patient only showed a response against the GT1a sequence. No responses against the GT1b variant were detected.

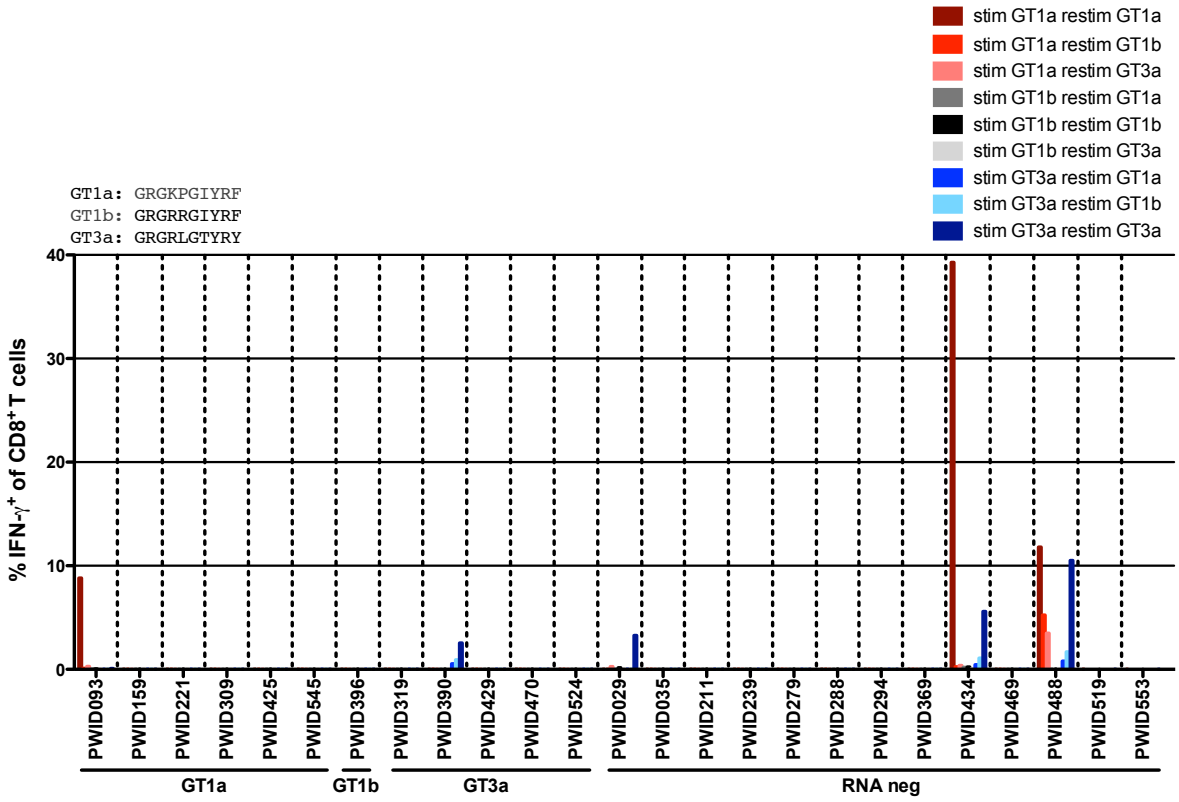

Figure 4.14: CD8$^+$ T cell response against the HLA-B*27-restricted epitope NS3$_{1492}$

The relative frequency of CD8$^+$IFN-γ$^+$ T cells after 10 days of *in vitro* expansion in the presence of the HLA-B*27-restricted peptides NS3$_{1492}$ was examined in 25 HLA-B*27 positive, HCV seropositive PWID via ICS and flow cytometric analysis. Cross-reactivity of CD8$^+$ T cells was evaluated by ICS for IFN-γ after restimulation with either the GT1a, GT1b or GT3a peptide. Each bar represents the CD8$^+$ T cell response to one peptide.

As a second example we wanted to examine the HLA-B*27 restricted immunodominant epitope NS5B$_{2841}$. Even though a similar study did not find any responses against the GT3a variant of this epitope (Neumann-Haefelin et al., 2010), we found two relatively weak responses against this epitope during our initial screen in GT3a infected PWID (see figure 4.6). As the study by Neumann-Haefelin *et al*. did not include patients who had resolved a GT3a infection, we wanted to have a closer look at this epitope in our PWID cohort, in which patients are regularly exposed to both genotypes.

CD8$^+$ T cell responses against the GT1 variant of the epitope were detected in 14 PWID, while 10 PWID showed some sort of response against the GT3 variant of this epitope (figure 4.15). In most cases, the GT3 response was a lot weaker than the

GT1 response and was probably caused by cross-reactivity of the GT1 response. In contrast to this, in four patients a response of nearly equal or even greater magnitude was detected against the GT3 sequence. Interestingly, these patients were either chronically GT3a infected (PWID470) or showed evidence for a previously resolved GT3a infection in the form of $CD8^+$ T cell responses against other GT3a-specific responses (either $NS3_{1492}$ (see figure 4.14), $NS2_{840}$ or $NS2_{942}$ (data not shown)).

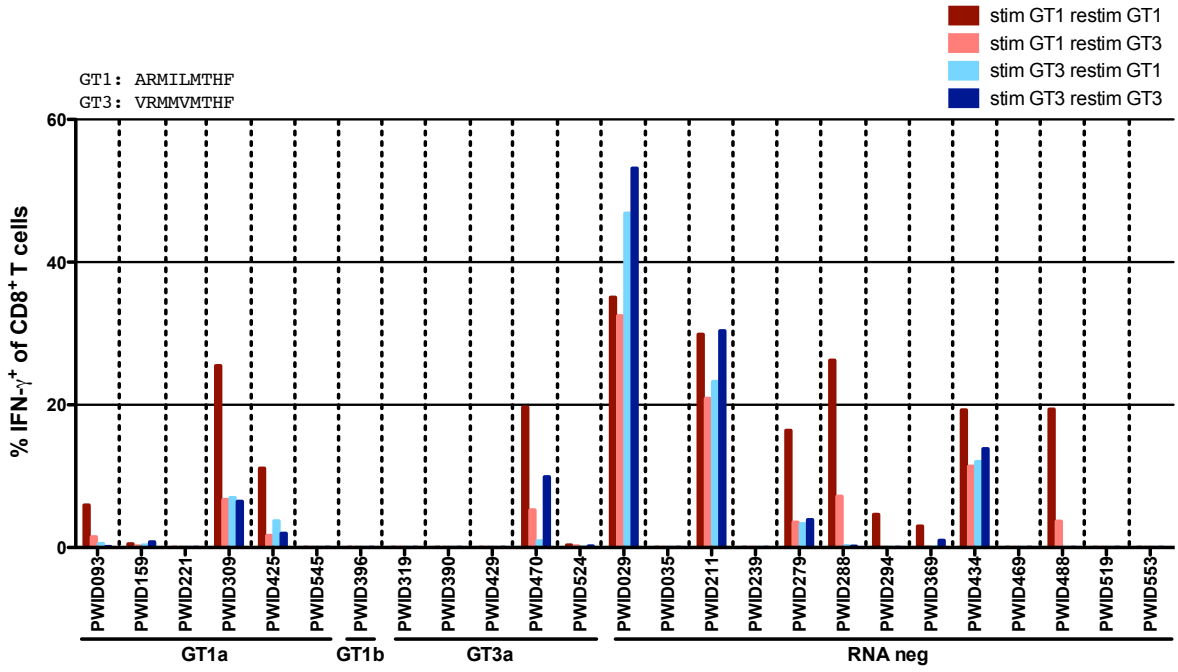

Figure 4.15: $CD8^+$ T cell response against the HLA-B*27-restricted epitope $NS5B_{2841}$

The relative frequency of $CD8^+IFN\text{-}\gamma^+$ T cells after 10 days of *in vitro* expansion in the presence of the HLA-B*27-restricted peptides $NS5B_{2841}$ was examined in 25 HLA-B*27 positive, HCV seropositive PWID via ICS and flow cytometric analysis. Cross-reactivity of $CD8^+$ T cells was evaluated by ICS for IFN-γ after restimulation with either the GT1 or GT3 peptide. Each bar represents the $CD8^+$ T cell response to one peptide.

4.4.2 HLA-B*13 restricted epitope $NS3_{1627}$

As a next example the previously described $CD8^+$ T cell immune response against the immunodominant HLA-B*13-restricted $NS3_{1627}$ was analysed in 18 HLA-B*13 positive PWID. For this epitope the GT1a and GT1b sequences are identical (RLGAVQNEV, labelled GT1) and only the GT3a sequence shows any sequence variation (RLGPVQNEI, labelled GT3).

A response against either one or both genotype-specific peptides could be detected in 12 out of 18 patients (figure 4.16). Half of the tested patients displayed two distinct T cell population directed against both genotypes, even though the strength of the

two responses differed widely between the patients from barely detectable for one genotype to equal in strength for both genotypes. In GT1 infected patients and in patients with resolved HCV infection 3 out of 6 patients exhibited $CD8^+$ T cell responses against either of the variant epitopes, while at least one of the responses could be detected in all GT3 infected patients.

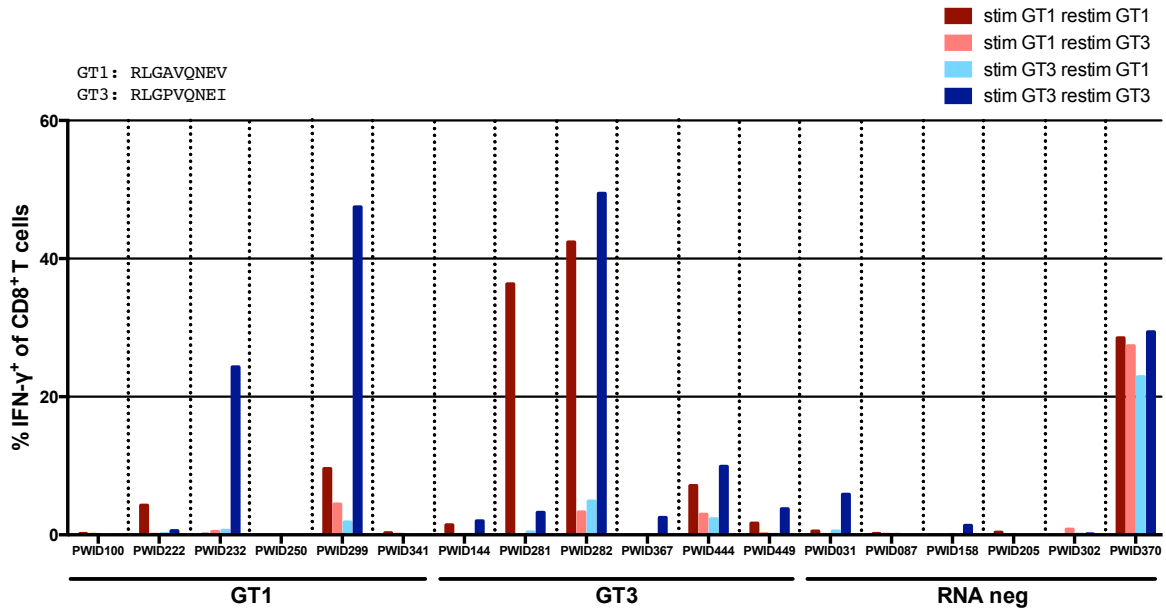

Figure 4.16: $CD8^+$ T cell response against the HLA-B*13-restricted epitope $NS3_{1627}$

The relative frequency of $CD8^+IFN\text{-}\gamma^+$ T cells after 10 days of *in vitro* expansion in the presence of the HLA-B*13-restricted peptides $NS3_{1627}$ was examined in 18 HLA-B*13 positive, HCV seropositive PWID via ICS and flow cytometric analysis. Cross-reactivity of $CD8^+$ T cells was evaluated by ICS for IFN-γ after restimulation with either the GT1 or GT3 peptide. Each bar represents the $CD8^+$ T cell response to one peptide.

Surprisingly, the most responses against both genotypes could also be found in GT3 infected subjects (5 out of 6), while only 2 out of 6 subjects each had a response against both genotypes in the GT1 infected PWID and PWID with resolved infection.
In the majority of cases these T cells were either not cross-reactive at all or only cross-reactive to a very small degree. The only exception to this observation was PWID 370, whose PBMCs were fully cross-reactive between both genotypes.
As a next step we wanted to show that the two genotype-specific responses within one subject actually represent two distinct T cell populations that arose from different precursor cells. For this purpose all subjects with at least one reponse against one of the HLA-B*13 epitope variants were analysed for their TCR Vβ chain usage via flow cytometric analysis (figure 4.17A).

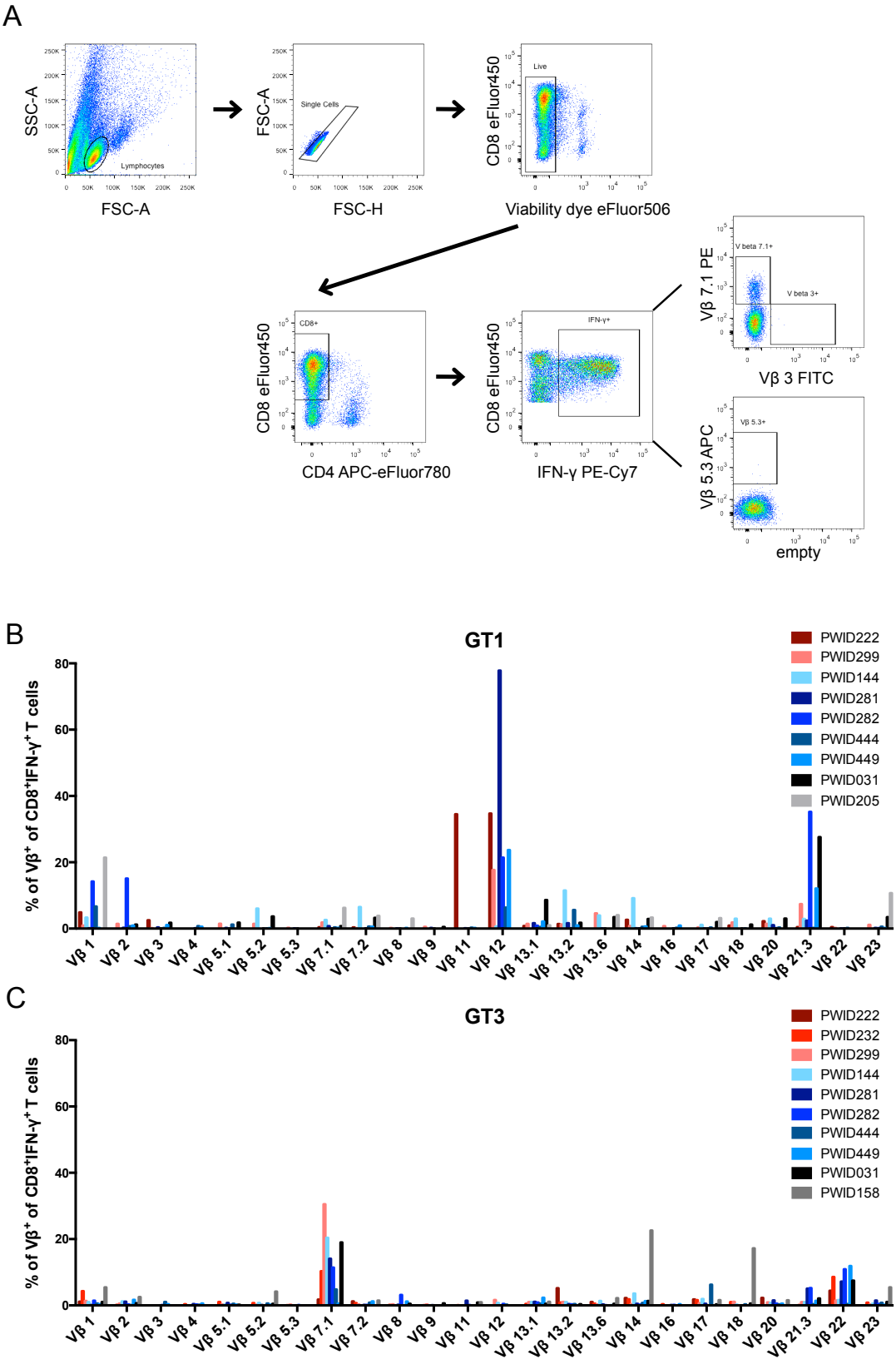

Figure 4.17: Vβ repertoire of HLA-B*13 NS3$_{1627}$-specific CD8$^+$ T cells

PWID with positive IFN-γ responses against GT1 or GT3 were further analysed for their TCR Vβ chain usage via flow cytometry. **A** Gating strategy for identification of HCV-specific CD8$^+$ T cells with certain Vβ chains. Gating for Vβ pool 1 is shown as an example. **B** Vβ repertoire of RLGAVQNEV (GT1)-specific CD8$^+$ T cells. **C** Vβ repertoire of RLGPVQNEI (GT3)-specific CD8$^+$ T cells.

The subjects that had a response against the GT1 epitope mainly displayed Vβ1, Vβ2, Vβ11, Vβ12 and Vβ21.3 on their surface (figure 4.17B), whereas subjects with a GT3-specific response mainly displayed Vβ7.1, Vβ14, Vβ16 and Vβ22 on their surface (figure 4.17C). Taken together these data indicate that the $CD8^+$ T cells recognising the two different genotype-specific variants of the HLA-B*13 epitope use distinct TCR Vβ repertoires.

4.4.3 HLA-B*15-restricted epitope $NS5B_{2466}$

Subsequently, we tested the $CD8^+$ T cell response against the HLA-B*15 restricted epitope $NS5B_{2466}$. For this epitope the viral consensus sequence differs not only between GT1 and GT3, but also between the subtypes GT1a and GT1b. Therefore all 9 possible combinations were tested for their respective cross-reactivity (figure 4.18).

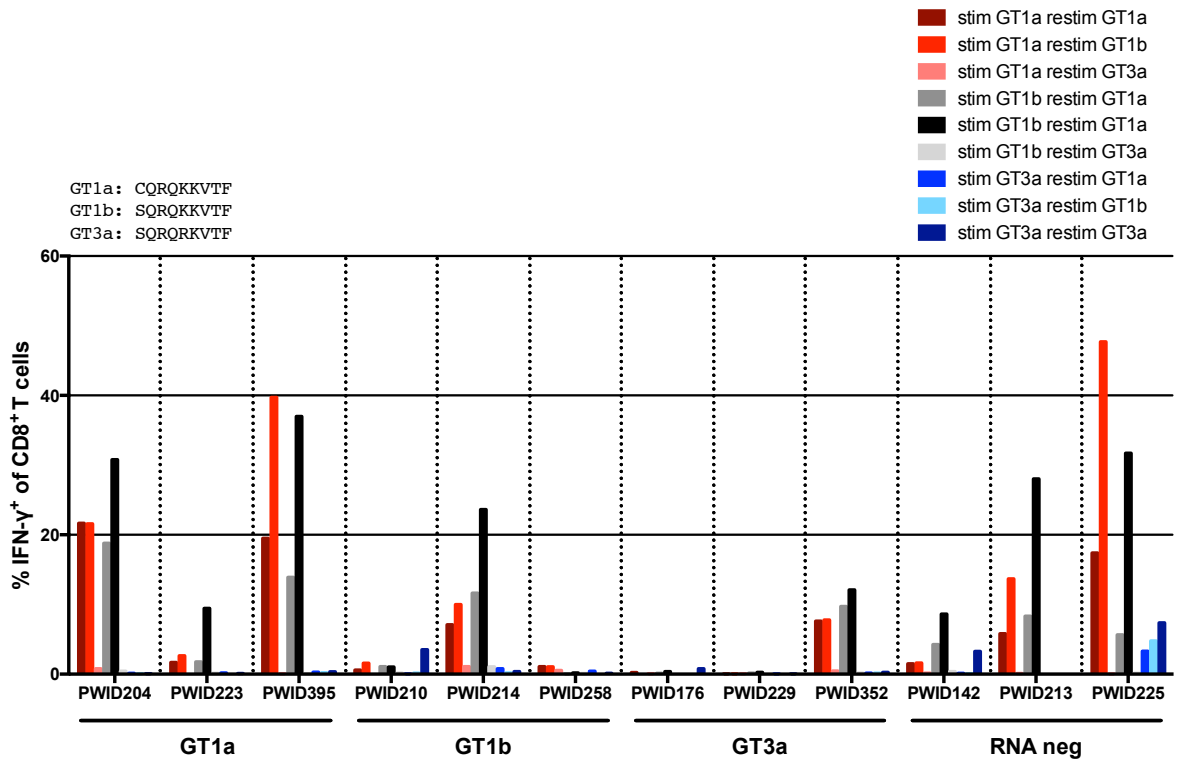

Figure 4.18: $CD8^+$ T cell response against the HLA-B*15-restricted epitope $NS5B_{2466}$

The relative frequency of $CD8^+IFN\text{-}\gamma^+$ T cells after 10 days of *in vitro* expansion in the presence of the HLA-B*15-restricted peptides $NS5B_{2466}$ was examined in 12 HLA-B*15 positive, HCV seropositive PWID via ICS and flow cytometric analysis. Cross-reactivity of $CD8^+$ T cells was evaluated by ICS for IFN-γ after restimulation with either the GT1a, GT1b or GT3a peptide. Each bar represents the $CD8^+$ T cell response to one peptide.

$CD8^+$ T cell responses could mainly be detected against GT1a and GT1b. The GT1b-specific response was the predominant one, which could be observed in 8 out of 12 patients, while a GT1a-specific response was detectable in 6 patients. GT1a-specific responses were only observed in conjunction with GT1b-specific responses. In addition, these responses were fully cross-reactive between GT1a and GT1b, but the GT1b response was usually stronger. The detected GT3a-specific responses were relatively weak and were only found in three patients. No cross-reactivity was observed between GT1-specific cells and the GT3a epitope and GT3a-specific $CD8^+$ T cells from only one patient (PWID225) showed minimal cross-reactivity with both GT1 epitopes.

To further analyse these different genotype- and subtype-specific responses, the TCR Vβ chain usage in patients with $CD8^+$ T cell responses against more than one genotype variant were determined via flow cytometry (figure 4.19).

Within one patient the Vβ chains used in GT1a and GT1b-specific cells were always identical, even if the usage differed between patients. For example, Vβ3 (figure 4.19A), Vβ5.1 and Vβ20 (figure 4.19B), V β14 (figure 4.19C) and Vβ17 (figure 4.19D) were observed on GT1a- and GT1b-specific cells in different patients. The presence of the same Vβ chains on both GT1-specific cells within each patient might be another indicator for the cross-reactivity of these cells.

The Vβ repertoire of all three genotypes could only be compared for PWID225 (figure 4.19D). For this patient the GT1a- and GT1b-specific $CD8^+$ T cells nearly exclusively displayed Vβ17 on their surface. This was only true for about 25% of GT3a-specific cells, while Vβ13.2 was present on another 25% of GT3a-specific cells. The presence of Vβ17 on $CD8^+$ T cells recognising all three genotypes might explain the slight cross-reactivity the GT3-specific cells exhibited towards the GT1 epitopes.

Overall, subtype-specific GT1 responses were largely cross-reactive and were recognised by TCRs using the same Vβ chains, while the GT3 response was not cross-reactive with either GT1 response and the corresponding TCR used a different Vβ chain.

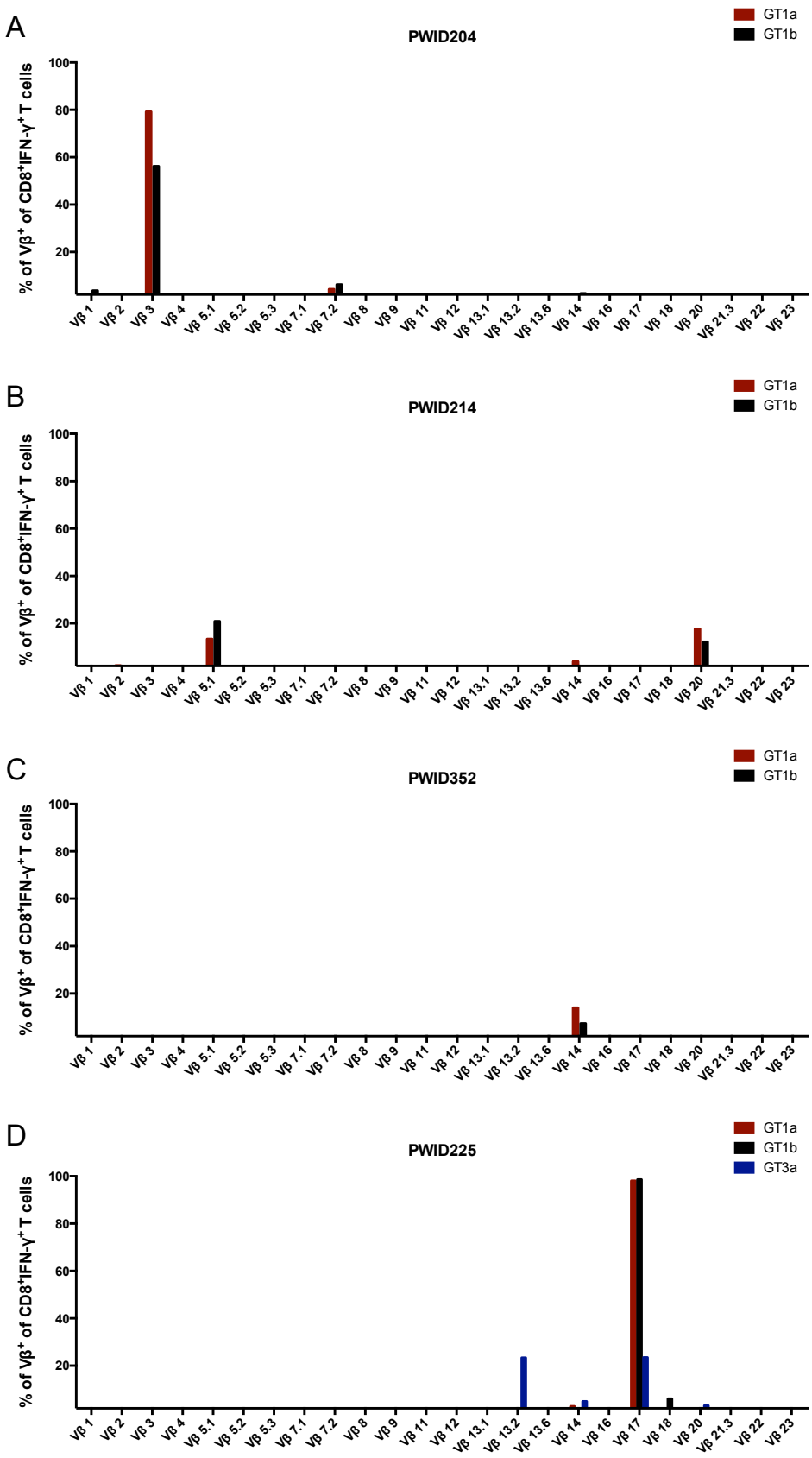

Figure 4.19: Vβ repertoire of HLA-B*15 NS5B$_{2466}$-specific CD8$^+$ T cells

PWID with positive IFN-γ responses against two or more epitope variants were further analysed for their TCR Vβ chain usage on CD8$^+$IFN-γ$^+$ T cells via flow cytometry. One example from each subgroup is shown (**A-D**).

4.4.4 HLA-B*57-restricted epitope NS5B$_{2629}$

As a last example, an HLA-B*57-restricted epitopes situated at position NS5B$_{2629}$ was analysed. The detected CD8$^+$ T cell responses were mainly directed against GT1a (6 out of 10 patients; figure 4.20). In four patients a GT1b-specific response could be detected in addition to the GT1a response, while three patients also had GT3a-specific responses.

The GT1b responses were always to some extend cross-reactive with the GT1a epitope and vice versa, but no cross-reactivity could be detected between the GT3a-specific responses and the GT1 epitopes in two of the patients (PWID370 and PWID376). Only PWID114 showed minimal cross-reactivity between all of the tested genotype-specific epitopes.

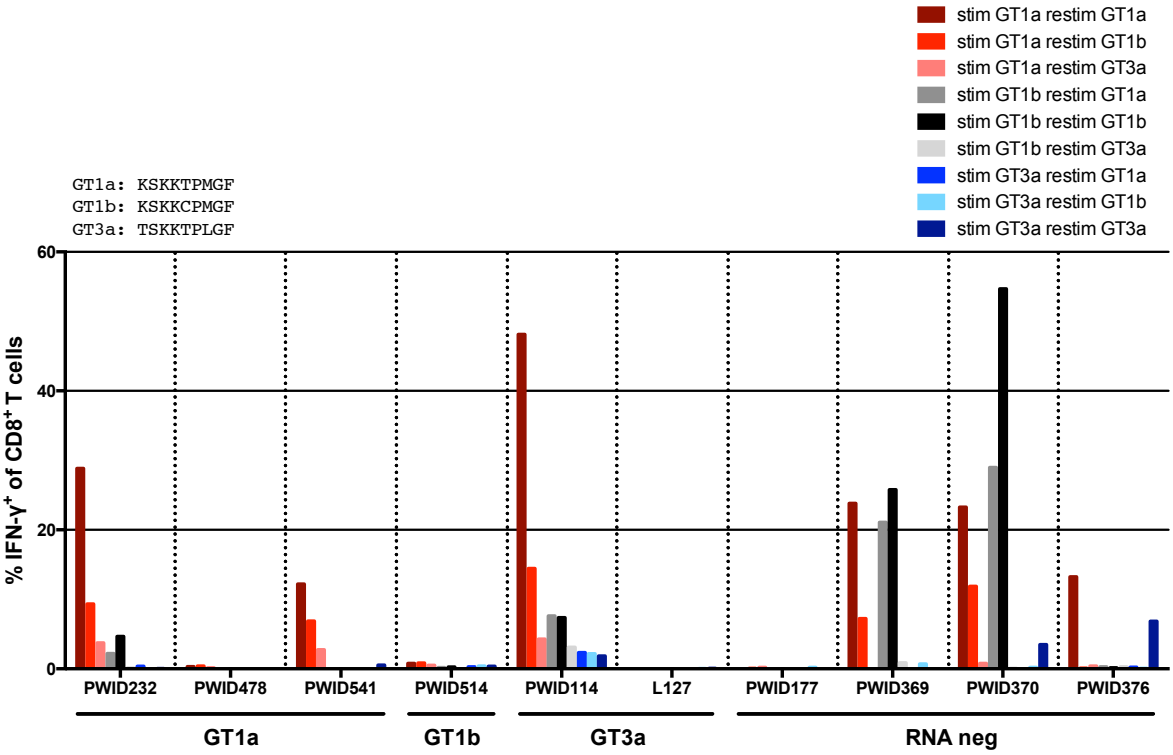

Figure 4.20: CD8$^+$ T cell response against the HLA-B*57-restricted epitope NS5B$_{2629}$

The relative frequency of CD8$^+$IFN-γ$^+$ T cells after 10 days of *in vitro* expansion in the presence of the HLA-B*57-restricted peptide NS5B$_{2629}$ was examined in 10 HLA-B*57 positive, HCV seropositive PWID via ICS and flow cytometric analysis. Cross-reactivity of CD8$^+$ T cells was evaluated by ICS for IFN-γ after restimulation with either the GT1a, GT1b or GT3a peptide. Each bar represents the CD8$^+$ T cell response to one peptide.

To determine whether the GT1a- and GT3a-specific CD8$^+$ T cell responses constitute two distinct T cell populations, the Vβ repertoire of these cells was analysed. As can be seen in figure 4.21, there is no overlap in the Vβ chains present on the surface of

GT1a- and GT3a-specific cells in either patient. The Vβ chain usage also differed between the two patients, although Vβ7.2 and Vβ22 were present on the surface of GT1a-specific cells in both patients.

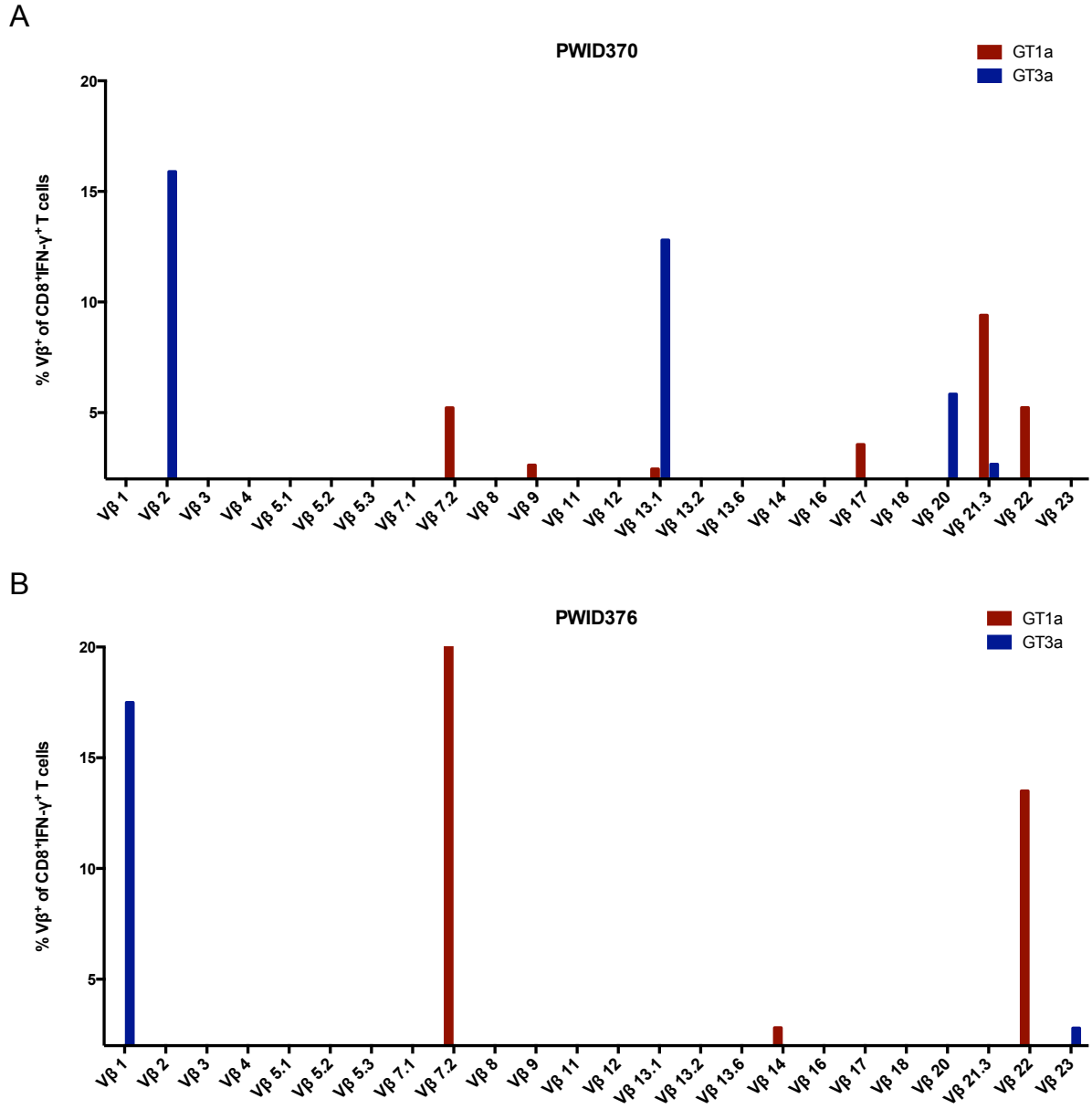

Figure 4.21: Vβ repertoire of HLA-B*57 NS5B$_{2629}$-specific CD8$^+$ T cells

PWID with positive IFN-γ responses against both GT1a and GT3a were further analysed for their TCR Vβ chain usage via flow cytometry. Vβ repertoire of PWID370 (**A**) and PWID376 (**B**) are shown for KSKKTPMGF (GT1a)-specific CD8$^+$ T cells and TSKKTPLGF (GT3a)-specific CD8$^+$ T cells.

In summary, not cross-reactive responses against two GT-specific variants were found for 4 out of 5 tested epitopes, while one epitope was cross-reactive between all tested GT variants (NS5B$_{2841}$). TCR Vβ chain analysis confirmed, that in most cases distinct CD8$^+$ T cell population were activated by the genotype-specific variants.

When the responses against all tested epitopes are summarised (figure 4.22), no significant differences in the recognition of the tested epitopes could be detected between the different PWID subgroups, although there was a slight trend towards more responses against both genotypes in GT3 infected and RNA negative PWID compared to GT1 infected PWID.

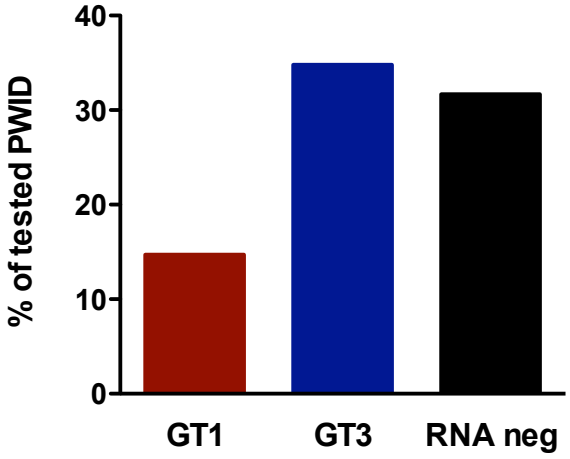

Figure 4.22: PWID with CD8$^+$ T cell responses against different variants of the same epitope

The number of responses against both genotypes from the ICS data for all 5 tested epitopes were added together and the percentage of total tested PWID within each subgroup (GT1 infected, GT3 infected or RNA neg) was calculated. Statistical significance was tested using Fisher's exact test.

Overall, we could show that priming against two different GT-specific variants of the same epitope is possible within one patient for most epitopes, even though these responses are not more frequently observed in PWID with resolved infection.

4.5 Depletion of HCV-specific CD8$^+$ T cells

Another factor influencing the outcome of HCV infection is exhaustion or outright depletion of HCV-specific CD8$^+$ T cells. To investigate if HCV-specific cells are in any way deleted in chronically infected patients, we first looked at the frequency of these cells directly after thawing and after 7 days of peptide-specific expansion via MHC class I dextramer staining (figure 4.23) in a preselected group with known responses against immunodominant CD8$^+$ T cell epitopes.

On day 0 HCV-specific cells were significantly less frequent in chronically infected patients compared to patients with resolved infection (p=0.0233, figure 4.23A). When the chronic subgroup was further subdivided into GT1a and GT3a infected patients, the GT1a infected subjects had even lower frequencies of HCV-specific cells, while GT3a infected patients displayed a frequency that was relatively close to patients with resolved infection. This difference in frequency between the genotypes can probably be explained by the fact, that the tested dextramer sequences were predominantly GT1-specific, (5 out of 7, see table 2.3). Consequently, the majority of responses detected in GT3a infected patients were probably due to a previously resolved GT1a infection. On day 7 after peptide specific expansion, no differences in the frequencies of HCV-specific cells could be detected between the three subgroups (figure 4.23B).

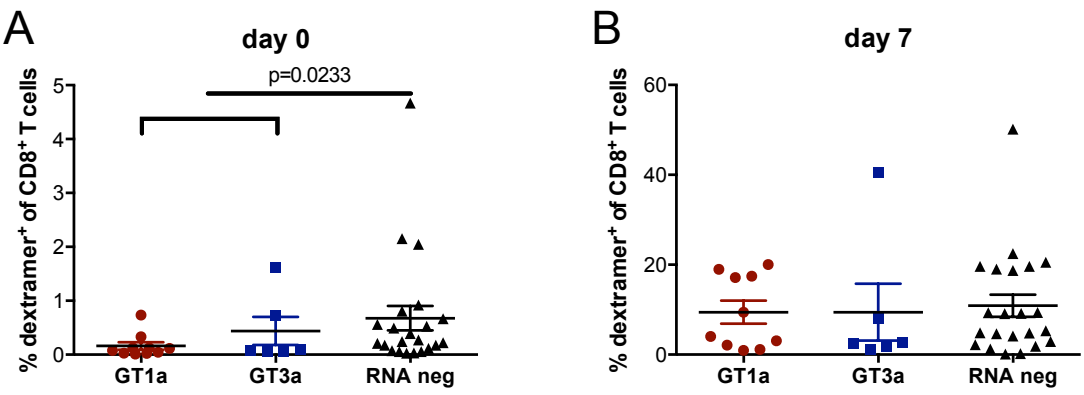

Figure 4.23: Frequency of HCV-specific CD8$^+$ T cells

The frequency of HCV-specific CD8$^+$ T cells was determined via MHC class I dextramer staining directly after thawing (**A**) and after 7 days of peptide specific expansion (**B**). p-values were calculated using Mann-Whitney test.

Next we wanted to check whether the autologous viral sequence matched the peptide sequence contained in the dextramers. Consequently, the viral sequences of

the chronically infected patients were determined (table 4.7). Except for one case, the viral sequence did not match the dextramer sequence.

Table 4.7 Cross-reactivity between autologous viral sequence and peptide sequence

ID	infecting genotype	peptide sequence	autologous viral sequence	cross-reactivity
PWID153	1a	ALYDVVTKL	ALYDVV**S**KL	yes
PWID153	1a	GVDPNIRTGV	GV**E**PNIRTGV	no
PWID160	1a	CINGVCWTV	CINGVCW**SI**	no
PWID160	1a	ALYDVVTKL	ALYDVV**G**KL	no
PWID174	3a	ATDALMTGY	ATDALMTG**F**	no*
PWID187	1a	ATDALMTGY	ATDALMTG**F**	no*
PWID197	3a	KLVALGINAV	KL**AGM**G**L**NAV	no
PWID197	3a	CINGVCWTV	**TVG**GV**M**WTV	no
PWID204	1a	ARMILMTHF	**V**RMIL**L**THF	no*
PWID206	3a	IPFYGKAI	IPFYGKA**L**	no
PWID274	3a	IPFYGKAI	**V**PFYGKAI	partial
PWID280	1a	CINGVCWTV	CINGVCWTV	-
PWID280	1a	KLVALGINAV	KLVALG**V**NAV	n.d.
PWID299	1a	KLVALGINAV	KLV**T**LGINAV	no
PWID347	3a	ATDALMTGY	ATDALMTG**F**	no*

*cross-reactivity was not tested for this patient, but similar studies have been published previously (Dazert et al., 2009; Neumann-Haefelin et al., 2008a); amino acids that differ between the dextramer sequence and the autologous viral sequence are marked in red; n.d.: not done.

As a next step the cross-reactivity between the autologous viral sequence and the dextramer sequence was determined via ICS to investigate if these substitutions constitute escape mutations. Only one case of full cross-reactivity and one case of partial cross-reactivity were found. In all other cases no cross-reactivity between the autologous viral sequence and the dextramer sequence could be detected indicating that the viral sequence could no longer be recognised and that these substitutions therefore constitute viral escape mutations.

As the presence of escape mutation in nearly all targeted epitopes with detectable responses does not adequately explain the differences in the frequencies of HCV-specific cells between GT1a and GT3a, we wanted to determine whether the depletion of HCV-specific cells in chronic PWID could be due to premature apoptosis of these cells. For this purpose the expression of the pro-apoptotic marker Bim was examined on these HCV-specific $CD8^+$ T cells directly after thawing and after 7 days of expansion (figure 4.24A and B). Bim has been previously shown to be upregulated

on hepatitis B virus (HBV)-specific CD8$^+$ T cells, probably caused by their antigen-specific activation in the liver compartment (Lopes et al., 2008).

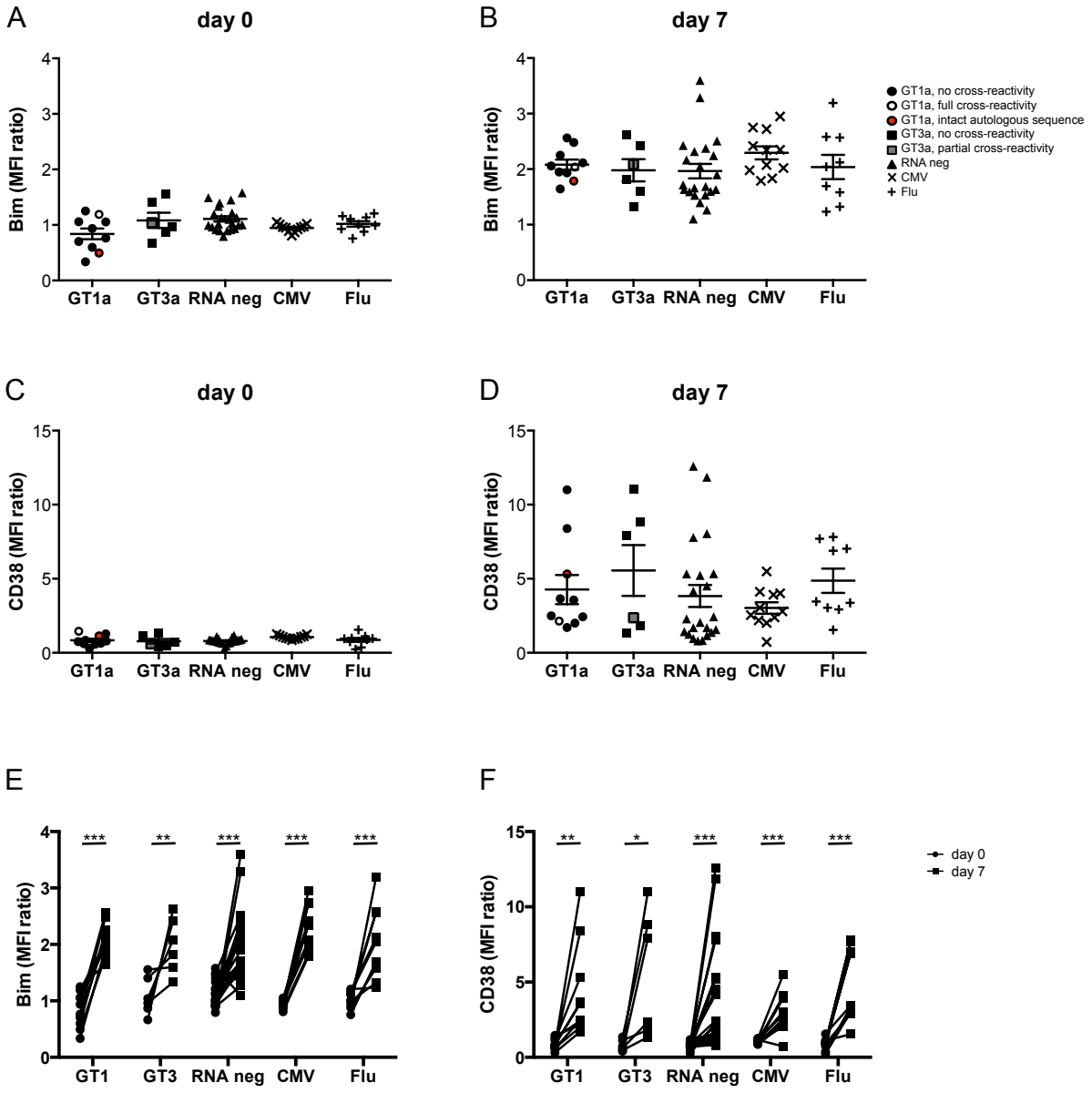

Figure 4.24: Expression of Bim and CD38 on HCV-specific CD8$^+$ T cells

The expression of Bim and CD38 on HCV-specific CD8$^+$ T cells was determined via flow cytometry. Median florescence intensity (MFI) ratios of Bim (**A**, **B** and **E**) and CD38 (**C, D** and **F**) expression was calculated by dividing the MFI value of the HCV-specific CD8$^+$ T cell population by the MFI value of the remaining CD8$^+$ T cell population. Horizontal lines indicate the mean. p-values were calculated using Student's t test (*:p>0.05, **: p>0.01 ***: p>0.001).

However, no differences in the expression of Bim was observed on HCV-specific CD8$^+$ T cells at day 0 or day 7 between chronically HCV infected PWID and HCV-RNA negative PWID (figure 4.24A and B). Moreover, there was also no difference in

Bim expression between HCV-specific $CD8^+$ T cells and CMV- or Flu-specific $CD8^+$ T cells. Even in the few cases where continuous antigen stimulation was expected based on the cross-reactivity profiles of the autologous epitope sequences, there were still no significant differences in Bim expression. Similar Bim levels were observed in $CD8^+$ T cells that did not recognise the autologous viral sequence (shown in black in figure 4.24) and in those with full or partial recognition (shown in gray or white). Of note, the single patient harbouring a virus with the prototype sequence in the targeted epitope also did not show differential Bim expression (shown in red). Upon antigen-stimulation Bim expression significantly increased by day 7 (figure 4.24E). The same results were observed for the activation marker CD38 (figure 4.24F). These findings indicate, that not only CD38 but also Bim is upregulated upon activation of $CD8^+$ T cells. Taken together, we do not find evidence that Bim is upregulated on HCV-specific $CD8^+$ T cells during chronic infection in PWID.

5 Discussion

PWID are the main risk group for acquiring HCV infection in Germany and other developed countries (Aceijas and Rhodes, 2007; Cornberg et al., 2011; Nelson et al., 2011). Due to their frequent risk behaviour of sharing needles and other injection equipment, they are likely to be repeatedly exposed to HCV. In our cohort from Essen GT1 and GT3 are the predominant genotypes and the distribution of genotypes is similar to those reported in other PWIDs in Germany (Zimmermann, 2012). Exposure to both genotypes is therefore highly probable for these patients. In this study we wanted to investigate the influence of the infecting viral genotype on the antiviral immune response with a particular focus on $CD8^+$ T cells.

5.1 Genetic host factors

5.1.1 *IFNL3* genotype

The C/C genotype of the SNP rs12979860 upstream of the *IFNL3* gene was first identified in a genome wide association study and was strongly associated with spontaneous clearance of HCV infection and enhanced response to treatment (Ge et al., 2009; Thomas et al., 2009). A role for this genetic marker in clearance of HCV could also be confirmed in other studies of cohorts that were mainly infected with GT1 (Beinhardt et al., 2012; Rauch et al., 2010; Tillmann et al., 2010), while the *IFNL3* genotype was found to only play a minor role in viral clearance in cohorts infected with GT2 or GT3 (reviewed in (Lange and Zeuzem, 2011)). This was in line with our data, as an increased frequency of the protective C/C genotype was only observed in patients with resolved HCV infection when compared to GT1 infected patients. The difference was far less pronounced when compared to GT3 infected patients.

It has been shown that the SNPs near this gene locus affect the expression of IFNL3, with the rs12979860 C/C genotype resulting in higher expression of IFNL3 within the liver compared to the T/T genotype (Dill et al., 2011), but the precise mechanism behind the protective effect has not been elucidated yet. The protective C/C genotype has also been linked to symptomatic hepatitis and jaundice, thereby indicating increased cytotoxic activity in the liver that might be caused by $CD8^+$ T cells. In addition, it has been previously described that IFNL3 could augment $CD8^+$ T

cell cytotoxicity and memory responses in macaques vaccinated with HIV antigen (Morrow et al., 2010) as well as enhance the adaptive immune response after vaccination in mice (Morrow et al., 2009). One small study of 53 Italian patients has even found a correlation between stronger and broader cell-mediated immunity and the rs12979860 C/C genotype, but no differentiation between $CD4^+$ and $CD8^+$ T cells was implemented (Spada et al., 2013).

We first analysed the $CD8^+$ T cell response in 115 PWID with HLA-matched optimal peptides. In line with other cross-sectional studies, PWID with resolved HCV infection had a significantly greater magnitude of the $CD8^+$ T cell response compared to PWID with chronic infection (Giugliano et al., 2009; Lauer et al., 2004), while the breadth of the response was also increased, but did not reach significant levels. In this study the percentage of recognised epitopes out of all tested epitopes was used as an indicator for the breadth of the response. Although this approach does not allow insights into the full epitope repertoire, it can still be used to estimate to what degree the "potential" repertoire of known $CD8^+$ T cell responses was utilised within each patient. Interestingly, there was no difference in the $CD8^+$ T cell response between GT1 and GT3 infected patients, even though the tested epitopes corresponded mostly to the GT1 sequence and sequence differences are known to prevent cross-recognition of most epitopes (Giugliano et al., 2009). The majority of the detected responses in GT3 infected PWID therefore probably stem from memory responses from a previously resolved GT1 infection.

To test the hypothesis that the *IFNL3* genotype influences the magnitude of the $CD8^+$ T cell response, this data set was stratified for the *IFNL3* genotype to determine a possible influence on the $CD8^+$ T cell response. However, no differences in either the magnitude or the breadth of the $CD8^+$ T cell response could be detected between patients with the C/C and non-C/C genotypes indicating that the $CD8^+$ T cell response was independent of the *IFNL3* genotype.

One possible limitation of the study is its cross-sectional design. In longitudinal studies in patients with acute HCV infection it has been shown that the magnitude of the $CD8^+$ T cell response does not differ between patients with spontaneous control of HCV replication and those that go on to develop chronic HCV infection suggesting that functional differences in HCV-specific T cells may be important during the acute phase (Cox et al., 2005a). Whether IFNL3 might still influence these functional differences has to be determined in longitudinal studies in larger cohorts.

5.1.2 HLA class I alleles

The HLA class I locus is the most polymorphic gene in the entire human genome (Goulder and Walker, 2012; Robinson et al., 2011) and the products of this gene locus, HLA-A, HLA-B and HLA-C molecules, have been shown to be associated with differential outcome of HCV infection in several studies. As $CD8^+$ T cell responses are important for viral clearance (Bowen and Walker, 2005) and HLA molecules determine which epitope can be presented to $CD8^+$ T cells in order to elicit a response, this association is not surprising. In particular, HLA-A*03, HLA-B*27, HLA-B*57 and HLA-C*01 have been associated with viral clearance (Kim et al., 2011; Kuniholm et al., 2010; McKiernan et al., 2004), while HLA-B*08, HLA-B*18 and HLA-C*04 have been linked to persistent infection (Kim et al., 2011; McKiernan et al., 2004; Thio et al., 2002).

Nevertheless, the data on which HLA class I alleles are associated with HCV infection outcome is still conflicted, because different studies found different HLA alleles to be associated with viral clearance. These differences can probably be attributed to differences in study populations as well as study design. For example, the studies by Thio *et al.*, Kim *et al.* and Kuniholm *et al.* all analysed multiracial cohorts and did not differentiate between different HCV genotypes, while the study by McKiernan *et al.* focused on a single-source GT1b outbreak in Irish women. Interestingly, another study of a similar single-source GT1b outbreak in German women found none of the associations between HLA alleles and infection outcome that were reported in the Irish cohort (Ziegler et al., 2013). The different findings in these two studies have been attributed to substitutions in immunodominant $CD8^+$ T cell epitopes in the German cohort. These findings show that sequence differences in immunodominant epitopes of the infecting virus can influence the outcome of HCV infection. Consequently, we wanted to test what influence different genotypes (GT1 and GT3 in particular) have on the association of certain HLA alleles with HCV infection outcome.

Our cohort consisted of 310 PWID from Essen. Even though the race/ethnicity of participating patients was not explicitly investigated, a Caucasian HLA background can be assumed for the majority of PWID, which is supported by the fact that the distribution of HLA alleles did not significantly differ between our study cohort and the general German population. The analysis of associations between HLA class I alleles and outcome of HCV infection in our PWID cohort only revealed two significant

results: HLA-B*08 was significantly more common in GT1 infected patients, but not in GT3 infected patients, compared to patients with resolved infection, while HLA-B*27 was significantly more frequent in patients with resolved infection compared to both GT1 and GT3 infected patients. Both observations are interesting in different ways. The HLA-B*08 association with viral persistence in GT1, but not GT3 shows that differences in the viral genotype can influence infection outcome. HLA-B*08 has also been associated with more rapid disease progression or increased susceptibility to infection in HIV (Carlson et al., 2012; Goulder and Walker, 2012; Pereyra et al., 2010; Steel et al., 1988) as well as a detrimental outcome during HCV infection in other studies that either included only GT1 infected patients (McKiernan et al., 2004) or a majority of GT1 infected patients (Kim et al., 2011), even though one study also reported it as a protective allele (Hraber et al., 2007). The results from this last study might be less reliable, as no information on the infecting viral genotype was available and chronically infected patients could only be distinguished from patients with resolved infection in a small minority of patients due to insufficient data. The mechanism behind this detrimental effect has not been studied yet, but a similar detrimental effect has been shown in HIV. The association of HLA-B*35:01 with rapid disease progression in B clade HIV infection has been linked to the absence of the immunodominant $CD8^+$ T cell epitope $Gag_{253-262}$, while no disease susceptibility has been found for C clade infection where this epitope is highly targeted even though the sequences between clades only differ in one amino acid residue (Gao et al., 2001; Lazaryan et al., 2011; Pereyra et al., 2010).

Finding a $CD8^+$ T cell epitope that could explain the detrimental effect of HLA-B*08 during HCV GT1 infection would require more extensive screening of $CD8^+$ T cell responses in GT3, because the known immunodominant HLA-B*08 epitope in GT1 ($NS3_{1395}$ HSKKKCDEL) has been shown to be cross-reactive with the corresponding GT3 epitope ($NS3_{1395}$ HSKKKCDEI) (Giugliano et al., 2009). Data on other $CD8^+$ T cell epitopes in GT3 is sparse, as only two studies to date have analysed the $CD8^+$ T cell response during GT3 infection: one study from our own lab that focused on responses against the NS3 protein and only found the before mentioned epitope (Giugliano et al., 2009) and a study by Humphreys *et al.* that did not narrow down the HLA-restriction for new epitopes in GT3 (Humphreys et al., 2012).

A protective effect of HLA-B*27 has been previously described for HIV (Goulder and Watkins, 2008; Kaslow et al., 1996; McNeil et al., 1996; Pereyra et al., 2010;

Trachtenberg et al., 2003) and for GT1b of HCV (McKiernan et al., 2004), but a second study by Neumann-Haefelin *et al.* concluded that this protective effect was limited to GT1 and would not be valid in GT3, because the immunodominant GT1 epitope $NS5B_{2841}$ ARMILMTHF, which was linked to the protective effect, is neither conserved nor effectively targeted in GT3 (Neumann-Haefelin et al., 2010). In addition, they also found HLA-B*27 to be significantly more frequent in GT3 infected patients compared to GT1 infected patients. This comparison is different from the one analysed in this project, as we mainly compared HLA allele frequencies between patients infected with either genotype to patients with spontaneously resolved infection, while any data on patients with resolved infection is missing in the other study. Consequently, conclusions with regard to the "protectiveness" of HLA-B*27 are limited when patients with spontaneous resolution of HCV-RNA were excluded from the analysis. The comparison of the frequency of HLA-B*27 between the two genotypes does not yield significant differences for genotype or phenotype frequencies in our cohort (p=0.7793 and p=0.7758, respectively; Fisher's exact test), even though very similar numbers of GT3 infected patients were included. The exact reasons for different results in the cohort from Freiburg and our cohort are unknown. One striking difference is that our cohort was exclusively comprised of PWID whereas the cohort from Freiburg included anti-HCV positive patients irrespective of the transmission route. Repetitive exposures to HCV as observed in PWID may be required for the protective effect of HLA-B*27 against HCV GT3 infection, however, this needs to be further addressed.

5.2 HLA-B*27-restricted HCV-specific $CD8^+$ T cell responses in GT3a

The known immunodominant epitope $NS5B_{2841}$ in GT1 has been shown not to be targeted in chronically GT3 infected patients by Neumann-Haefelin *et al.* (Neumann-Haefelin et al., 2010). Nevertheless, we were able to detect $CD8^+$ T cell responses against the GT3 sequence of nearly equal or even greater magnitude in four patients. This was exclusively in patients that showed evidence for a resolved GT3 infection in the form of other $CD8^+$ T cell responses that are only immunogenic in GT3a or not cross-reactive with GT1 or were chronically infected with GT3. The difference between our results and those described in the previous study can likely be explained by the inclusion of patients with resolved infection that have previously been infected with GT3, as $CD8^+$ T cell responses against this genotype would be

easier to detect in such patients. In the future a closer examination of these $NS5B_{2841}$-specific cells that are fully cross-reactive between GT1 and GT3 would be interesting, in particular with regard to their TCR sequence to see whether differences can be found between cells that do recognise the GT3 sequence and those that do not.

Overall the responses to the GT3 variant of the $NS5B_{2841}$ probably cannot explain the protective effect of HLA-B*27 we observed in our cohort, as strong responses only occurred in a small minority of patients. Therefore, we wanted to see whether we could identify novel $CD8^+$ T cell epitopes directed against GT3 that might not be immunogenic in GT1 due to the sequence differences between GT1 and GT3. A screen of predicted HLA-B*27:05 binding epitopes in GT3 after *in vitro* expansion revealed two novel vigorously targeted epitopes in NS2 ($NS2_{840}$ and $NS2_{942}$). These were confirmed to be strong binders in a binding assay involving UV-induced peptide-exchange reactions. Surprisingly, this assay also showed that only one third (18 out of 54 tested peptides) of predicted and known epitopes actually bound with high affinity to HLA-B*27:05 in an experimental setting indicating that computational models should not be used as the sole indicator for peptide-MHC binding affinity. High affinity binding to HLA-B*27:05 could be shown for nearly all peptides that elicited a response during our ICS screen, which is not surprising as a strong peptide-MHC binding is necessary to create an interaction with the TCR on the $CD8^+$ T cell.

Only the previously described epitope $p7_{780}$ had a low binding affinity to HLA-B*27:05. This epitope has only been described in a screen of one acutely infected patient (Neumann-Haefelin et al., 2006). This patient was of Sicilian ancestry and was later shown to carry not the most common HLA-B*27 subtype HLA-B*27:05, but the less frequent HLA-B*27:02 allele (Nitschke et al., 2014). Another epitope identified in this patient, $NS5B_{2820}$, has already been shown to be restricted by HLA-B*27:02 and not HLA-B*27:05. The same likely applies to the $p7_{780}$ epitope and would thereby explain the low binding affinity to HLA-B*27:05.

The two novel GT3 epitopes $NS2_{840}$ and $NS2_{942}$ were also confirmed to be vigorously targeted *ex vivo* via MHC class I dextramer stainings. Especially for the $NS2_{942}$ epitope the frequency of detection of specific $CD8^+$ T cells was nearly as high as for the known immunodominant GT1 epitope $NS5B_{2841}$ and the magnitude of the response was comparable as well. In addition, the $NS2_{942}$ epitope was targeted in the

majority of GT3 infected patients and patients with resolved infection. The $NS2_{840}$ epitope seemed to be subdominant, as it was targeted with reduced magnitude and in fewer patients compared to the $NS5B_{2841}$ and $NS2_{940}$ epitope.

When the $CD8^+$ T cell response against the GT1a and GT1b variants of the two NS2 epitopes were tested, no responses against the GT1 sequences were detected and no cross-reactivity between the GT3-specific responses and either of the GT1 variants could be detected, indicating that both novel epitopes are only immunogenic in GT3a, but not in GT1a or GT1b. This can probably be attributed to the fact that the HLA-B*27:05 binding anchor arginine at positions 2 of the epitope is not conserved in the majority of GT1 sequences.

No differences in the frequency of sequence polymorphisms could be observed in the subdominant $NS2_{840}$ epitope between GT3 infected patients according to their HLA-B*27 status. Additionally, the prototype sequence and the viral variants were all cross-reactive to some degree and responses against the viral variants could be detected in PWID with resolved infection.

In contrast to this, the biological relevance of the $CD8^+$ T cell response directed against the dominant $NS2_{942}$ epitope was further corroborated by the fact that sequence polymorphisms in this epitope in chronically GT3a infected patients were more likely to occur in HLA-B*27 positive patients than in HLA-B*27 negative patients. Furthermore, functional analysis revealed no $CD8^+$ T cell responses against the viral variants in the GT3a infected PWID harbouring the respective variant and no cross-reactivity between the prototype sequence and the viral variants could be detected in PWID with a response against the prototype sequence. Taken together our data indicate that HLA-B*27-mediated selection pressure on the $NS2_{942}$ epitope associated with viral escape occurs in GT3a infected patients. This is again similar to the observations made for the $NS5B_{2841}$ epitope in GT1, for which sequence differences between the prototype and the autologous viral sequence were also frequently found in HLA-B*27 positive patients, while the sequence was mostly intact in HLA-B*27 negative patients (Neumann-Haefelin et al., 2006). For this epitope the protective effect of HLA-B*27 has been attributed to the fact that several mutations inside this epitope are necessary to achieve viral escape, as $CD8^+$ T cells specific for the prototype sequence are otherwise able to cross-recognise the viral variants (Dazert et al., 2009), representing a relatively high genetic barrier to escape. This

mechanism of protection does probably not apply for the $NS2_{942}$ epitope, as one mutation was enough to abrogate recognition of the epitope by $CD8^+$ T cells.

Severe impairment of viral fitness by escape mutations have also been associated with the protective effect of two other HLA class I alleles: HLA-A*03 and HLA-B*57. In both cases secondary compensatory mutations were selected in patients carrying the respective allele as the primary escape mutations incurred high viral fitness costs (Fitzmaurice et al., 2011; Oniangue-Ndza et al., 2011). Unfortunately, similar experiments cannot be performed for GT3a at the moment, as no full-length replication model for HCV GT3a is available yet.

5.3 T cell responses targeting different variants of the same epitope

Due to sequence differences between the different HCV genotypes, $CD8^+$ T cell responses directed against a specific epitope are not necessarily cross-reactive. In a previous study from our group the co-existence of two distinct $CD8^+$ T cell populations directed against the both the GT1 and the GT3 sequence of the HLA-B*13 restricted epitope $NS3_{1627}$ that were not cross-reactive could be shown (Giugliano et al., 2009). In addition, responses against both genotype 1 and genotype 3 were more frequently observed in PWID with resolved infection, when both cross-reactive and distinct responses were considered together.

A similar effect has been observed during HIV infection, as an expansion of a $CD8^+$ T cell response against a related, but not cross-reactive variant epitope can occur after viral escape mutation in the corresponding $CD8^+$ T cell epitope (Allen et al., 2005; Roider et al., 2013). This ability to mount *de novo* responses to a variant epitope have been mainly described for $CD8^+$ T cell responses restricted by HLA alleles associated with a protective effect during HIV infection, e.g. HLA-B*27 and HLA-B*57 (Bailey et al., 2006; Feeney et al., 2005; Ladell et al., 2013; O'Connell et al., 2011).

Here we wanted to determine whether the co-existence of distinct $CD8^+$ T cell populations against both GT1 and GT3 restricted by HLA alleles that are associated with protection during either HIV or HCV infection (HLA-B*13, HLA-B*15, HLA-B*27 and HLA-B*57) occurs in PWID exposed to both genotypes. We tested 5 known immunodominant epitopes that differed in at least two amino acid positions between the GT1a or GT1b sequence and the GT3a sequence for the IFN-γ response of the $CD8^+$ T cells against the genotype-specific sequences as well as the cross-reactivity between all variant epitopes. $CD8^+$ T cell responses against GT1 and GT3 were

found in all 5 tested cases. In 4 cases these represented two distinct, not cross-reactive responses, while the responses against the HLA-B*27-restricted epitope $NS5B_{2841}$ were at least partially cross-reactive between the different genotypes.

When the responses against these epitopes were considered collectively, no evidence of a protective effect of the $CD8^+$ T cell responses targeting variants of the same epitope within one patient could be found, as there was no significant difference in the frequency between patients with chronic and resolved HCV infection.

Whether priming against two variants of the same epitope is possible has not been previously conclusively shown for HCV. Studies so far have only investigated whether $CD8^+$ T cells against escape variants can be primed during ongoing infection, but only sparse evidence for such populations was found (Salloum et al., 2008; Uebelhoer et al., 2008). In murine models it has been shown that secondary responses against distinct but closely related epitopes are impaired, a concept termed original antigenic sin (Klenerman and Zinkernagel, 1998), even though this is not a universal concept with regard to $CD8^+$ T cells (Zehn et al., 2010).

Here we show that priming of two distinct genotype-specific variants is possible for the majority of tested epitopes. The existence of these cells has important implications for vaccine design. If priming against two different variants of the same epitopes is possible within one patient, both could be included in a future vaccine thereby making it efficacious against several genotypes without having to rely on epitopes that are conserved between the different genotypes.

5.4 Depletion of HCV-specific $CD8^+$ T cells

$CD8^+$ T cell are known to play an important role in spontaneous clearance of acute HCV infection in both chimpanzee studies (Thimme et al., 2002) and humans (Thimme et al., 2001) as shown by the temporal association of their appearance with resolution of infection. Furthermore, during *in vivo* depletion experiments in chimpanzees, $CD8^+$ T cells have been shown to be the key effector cells controlling HCV replication (Shoukry et al., 2003). Exhaustion of these cells has been frequently observed in patients with chronic infection (Nakamoto et al., 2009; Radziewicz et al., 2007, 2008; Spangenberg et al., 2005; Wedemeyer et al., 2002) and exhausted $CD8^+$ T cells have been shown to be more prone to apoptosis linked to an upregulation of PD-1 expression (Radziewicz et al., 2007). In addition, decreased

frequencies of HCV-specific $CD8^+$ T cells have been frequently observed in chronically HCV infected individuals compared to patients who spontaneously resolve infection (Lauer et al., 2004), further indicating that apoptotic deletion of HCV-specific $CD8^+$ T cells might play a role in determining HCV infection outcome.

We analysed the *ex vivo* frequency of HCV-specific $CD8^+$ T cells in patients with either chronic or resolved infection via MHC class I dextramer stainings and found significantly lower frequencies in chronic patients compared to patients with resolved infection even though only patients with known responses against the tested epitopes were selected for analysis. A slight trend towards a lower frequency of HCV-specific $CD8^+$ T cells in GT1 infected patients compared to GT3 infected patients was observed, but did not reach statistical significance.

One possible explanation for the lower frequency of HCV-specific $CD8^+$ T cells in chronic patients is apoptotic deletion of this cell population. A possible mediator of apoptosis is the proapoptotic molecule Bim. This Bcl-2 family member is important for the regulation of T cell homeostasis, as it plays a central role in the initiation of apoptosis signalling in lymphocytes (Bouillet et al., 1999; O'Connor et al., 1998). In this context Bim is on the one hand important for the shutdown of $CD8^+$ T cell responses after acute viral infections to limit tissue damage (Pellegrini et al., 2003), while on the other hand downregulation of Bim is necessary to allow $CD8^+$ T cell memory formation (Sabbagh et al., 2006). In addition, Bim has been implicated with induction of apoptosis in $CD8^+$ T cells that have been activated in the liver in murine models (Holz et al., 2008). Bim has also been found to be upregulated in HBV-specific $CD8^+$ T cells from chronic HBV patients, which was again linked to their intrahepatic antigen-specific activation (Lopes et al., 2008). As the activation of HCV-specific $CD8^+$ T cells would also occur in the liver compartment, an involvement of Bim seems likely.

Bim expression has also previously been investigated in HCV-specific $CD8^+$ T cells (Larrubia et al., 2011, 2013), but no differences in Bim expression levels have been found in PBMC from patients with either chronic or spontaneously resolved HCV infection directly *ex vivo*. As both studies by Larrubia *et al.* were limited to two HLA-A*02 restricted epitopes in GT1 infected patients and did not take the influence of the autologous viral sequence on the exhaustion status of the $CD8^+$ T cells in chronic patients into account, we analysed the Bim expression in HCV-specific $CD8^+$ T cells from patients infected with either GT1 or GT3 using MHC class I dextramer restricted

by multiple HLA alleles. We could not find differential expression of Bim between patients with chronic and persistent HCV infection either. This effect might partially be explained by the fact that escape mutations were present in nearly all studied epitopes from chronically infected PWID, but Bim expression levels were not elevated when partial or full cross-reactivity with the autologous viral sequence could be shown or when the viral sequence matched the tested epitope sequence, either.

Another factor influencing our observation of lack of Bim involvement might be the fact that HCV-specific $CD8^+$ T cells in general (Neumann-Haefelin et al., 2008b) and those prone to apoptosis in particular (Radziewicz et al., 2008) have been shown to be more frequent in the liver compared to the peripheral blood. Consequently, Bim levels in HCV-specific $CD8^+$ T cells in the peripheral blood might differ from those sequestered in the liver. A comparison between HCV-specific $CD8^+$ T cells between both compartments would be interesting and necessary to exclude this possibility. Unfortunately, acquiring samples for this analysis is going to be difficult, as liver biopsy samples are not as easily obtained as blood samples and ethical concerns preclude such a procedure in patients with resolved HCV infection because there is usually no medical indication.

6 Summary

Transmission of hepatitis C virus (HCV) is closely linked to blood-blood contact. Accordingly, in developed countries HCV is mainly acquired by people who inject drugs (PWID). Importantly, PWID living in Germany are frequently exposed to different HCV genotypes (GTs) with GT1 and GT3 being predominant. As the infecting genotype can influence the infection outcome and HCV-specific $CD8^+$ T cells play a major role in the immune response against HCV, we wanted to investigate the influence of the viral GT on the antiviral $CD8^+$ T cell response in more detail in highly exposed individuals. For this purpose we worked with a cohort of 363 PWID from Essen, Germany that consisted of patients that remained seronegative for HCV (11%), patients with spontaneously resolved HCV infection (25%) and chronically infected patients (33% GT1, 27% GT3 and 4% other GTs).

As a first step, we examined two host genetic factors that have been implicated with affecting HCV infection outcome. Single nucleotide polymorphisms near the *IFNL3* gene have been associated with spontaneous clearance and enhanced response to therapy. In our cohort the "protective" *IFNL3* rs12979860 C/C GT was significantly less frequent in GT1 infected patients compared to patients with resolved HCV infection, while subjects infected with GT3 showed an intermediate GT frequency. No significant differences in the $CD8^+$ T cell response depending on the *IFNL3* GT were detected, suggesting that the protective effect mediated by the *IFNL3* C/C GT is independent of the $CD8^+$ T cell response.

Next, the impact of the HLA class I genotype on infection outcome was determined. In this cohort HLA-B*08 was a risk allele for GT1 infection, whereas HLA-B*27 was protective against both GT1 and GT3 infection. This second finding was unexpected, as HLA-B*27 was so far only described to protect against GT1 due to sequence differences in the immunodominant $CD8^+$ T cell epitope $NS5B_{2841}$ between both genotypes. We therefore aimed to identify novel GT3-specific $CD8^+$ T cell responses that might mediate the protective effect. By screening predicted HLA-B*27-binders we identified the two novel epitopes, $NS2_{840}$ GRLIWWNQY and $NS2_{942}$ GRWFNTYLY, with the latter one being immunodominant in GT3. The strength of the $CD8^+$ T cell response to the immunodominant GT3-specific B*27-epitope was comparable to the response against the known GT1-specific $NS5B_{2841}$ epitope. Viral sequence analysis of the NS2 epitope regions provided evidence for mutational

escape in HLA-B*27-positive patients in the $NS2_{942}$ epitope. In summary, different epitope repertoires could be detected in GT1 and GT3 infected patients indicating that different $CD8^+$ T cell responses contribute to the protective effect of HLA-B*27.

Genotype-specific differences in the elicited $CD8^+$ T cell response can also play an important role in determining the outcome of HCV infection. Our group has previously shown that patients with $CD8^+$ T cells active against both GT1 and GT3 are predominantly found in PWID with resolved infection. Here, we tested if distinct $CD8^+$ T cell responses against different sequence variants of the same epitope can be activated in patients exposed to different genotypes. The analysis was focused on immunodominant epitopes presented by HLA class I alleles that have been described in the context of immune control of viral infections (HLA-B*13, HLA-B*15, HLA-B*27 and HLA-B*57). Distinct responses against two GT-specific variants were found for 4 out of 5 tested epitopes, while one epitope was cross-reactive between all tested GT variants. Analysis of the T cell receptor (TCR) Vβ chain repertoire confirmed in most cases that distinct $CD8^+$ T cell populations were activated by the different variants. As a proof of principle, priming against two different GT-specific variants of the same epitope is therefore possible within one patient, suggesting that inclusion of different sequence variants of important epitopes in a prophylactic vaccine may be feasible.

As a last factor influencing the outcome of HCV infection in different genotypes we looked at the deletion of HCV-specific $CD8^+$ T cells, as a rapid loss of specific T cells is observed upon viral persistence and continuous antigen exposure. We confirmed lower frequencies of HCV-specific $CD8^+$ T cells in chronically infected patients compared to patients with resolved infection. It was tested whether the pro-apoptotic protein Bim is involved in $CD8^+$ T cell depletion in HCV, as it was reported that activation of T cells in the liver of mice and in human HBV infection leads to upregulation of Bim. In contrast to HBV, Bim was not differentially regulated in $CD8^+$ T cells from patients with resolved or chronic HCV infection *ex vivo*. Upon antigen-specific activation Bim and CD38 were both upregulated irrespective of the disease status. Taken together, our data suggest that dysregulation of Bim does not seem to contribute to $CD8^+$ T cell depletion during chronic HCV infection.

7 Zusammenfassung

Die Übertragung von Hepatitis C Virus (HCV) geschieht hauptsächlich über Blutkontakt, so dass intravenöse Drogengebraucher („people who inject drugs", PWID) die Hauptrisikogruppe für HCV Infektionen in entwickelten Ländern darstellen. PWID in Deutschland sind häufig unterschiedlichen HCV Genotypen (GT) ausgesetzt, wobei GT1 und GT3 am weitesten verbreitet sind. Da der infizierende Genotyp den Ausgang der Infektion beeinflussen kann und HCV-spezifische $CD8^+$ T Zellen eine wichtige Rolle bei der Immunantwort gegen HCV spielen, wollten wir im Detail untersuchen, welchen Einfluss der virale Genotyp auf die antivirale $CD8^+$ T Zellantwort in hochexponierten Individuen hat. Zu diesem Zweck wurde eine Kohorte von 363 PWID aus Essen, Deutschland untersucht, die aus seronegativen Patienten (11%), Patienten mit spontan ausgeheilter HCV Infektion (25%) und Patienten mit chronischer Infektion (33% GT1, 27% GT3 und 4% andere GT) bestand.

Zunächst wurden zwei genetische Wirtsfaktoren analysiert, die Auswirkungen auf den Ausgang einer HCV Infektion haben können. Einzelne Nukleotidpolymorphismen nahe dem *IFNL3* Gen wurden mit spontaner Ausheilung und besserem Ansprechen auf Therapie assoziiert. Verglichen mit Patienten mit ausgeheilter HCV Infektion kam der „protektive" *IFNL3* rs12979860 C/C GT in unserer Kohorte wesentlich weniger häufig in GT1 infizierten Patienten vor. Dahingegen hatten GT3 infizierte Patienten eine intermediäre GT Frequenz. Es wurden keine signifikanten Unterschiede in der $CD8^+$ T Zellantwort abhängig vom *IFNL3* GT gefunden. Dies deutet darauf hin, dass der protektive Effekt des *IFNL3* C/C GT nicht durch die $CD8^+$ T Zellantwort vermittelt wird.

Als nächstes wurde der Einfluss des HLA Klasse I Genotypen auf den Ausgang der Infektion ermittelt. In unserer Kohorte war HLA-B*08 ein Risikoallel, wohingegen HLA-B*27 protektiv gegen sowohl GT1 als auch GT3 war. Diese zweite Beobachtung war unerwartet, da der protektive Effekt von HLA-B*27 bis jetzt nur für GT1 beschrieben worden war. Dies wurde auf Sequenzunterschiede zwischen den Genotypen in dem immundominanten $CD8^+$ T Zellepitop $NS5B_{2841}$ zurückgeführt. Daher wollten wir neue GT3-spezifische $CD8^+$ T Zellantworten identifizieren, die diesen protektiven Effekt erklären könnten. Das Screening von vorausgesagten HLA-B*27 Bindern ergab zwei neue Epitope: $NS2_{840}$ GRLIWWNQY and $NS2_{942}$ GRWFNTYLY. Das $NS2_{942}$ Epitop war immundomiant in GT3 und die Stärke der

$CD8^+$ T Zellantwort war vergleichbar mit dem bekannten GT1-spezifischen $NS5B_{2841}$ Epitop. Virale Sequenzanalysen der NS2 Epitopregionen lieferten Beweise für Escapemutationen im $NS2_{942}$ Epitop. Zusammenfassend lässt sich sagen, dass verschiedene Epitoprepertoire in GT1 und GT3 infizierten Patienten vorhanden sind und daher verschiedene $CD8^+$ T Zellen zu dem protektiven Effekt von HLA-B*27 beitragen.

Genotyp-spezifische Unterschiede in der $CD8^+$ T Zellantwort können den Ausgang einer HCV Infektion erheblich beeinflussen. Unsere Arbeitsgruppe hat bereits zuvor gezeigt, dass Patienten mit $CD8^+$ T Zellantworten, die gegen GT1 und GT3 gerichtet sind, hauptsächlich in PWID mit ausgeheilter Infektion vorkommen. Daher wollten wir testen, ob Patienten, die verschiedenen Genotypen ausgesetzt sind, distinkte $CD8^+$ T Zellantworten gegen verschiedene Sequenzvarianten desselben Epitops besitzen. Die Analyse wurde auf immundominante Epitope konzentriert, die von HLA Klasse I Molekülen präsentiert werden, die im Zusammenhang mit Immunkontrolle von Virusinfektionen beschrieben wurden (HLA-B*13, HLA-B*15, HLA-B*27 und HLA-B*57). Distinkte Antworten gegen zwei GT-spezifische Varianten wurden in 4 von 5 Fällen gefunden, während ein Epitop kreuzreaktiv zwischen den getesteten GT Varianten war. Eine Analyse des T Zellrezeptor Vβ Kettenrepertoires bestätigte, dass verschiedene Varianten in den meisten Fällen distinkte $CD8^+$ T Zellpopulationen aktivieren. Dies zeigt, dass ein Priming gegen zwei verschiedene GT-spezifische Varianten desselben Epitops in einem Patienten möglich ist. Daher wäre die Aufnahme verschiedener Sequenzvarianten wichtiger Epitope in eine prophylaktische Vakzine sinnvoll.

Als letzter Faktor, der den Ausgang einer HCV Infektion beeinflussen kann, wurde die Deletion von HCV-spezifischen $CD8^+$ T Zellen untersucht, da ein rapider Verlust von spezifischen T Zellen bei viraler Persistenz und kontinuierlicher Antigenaussetzung beobachtet wird. Wir konnten niedrigere Frequenzen von HCV-spezifischen $CD8^+$ T Zellen in chronisch infizierten PWID bestätigen. Es wurde untersucht, ob das pro-apoptotische Protein Bim eine Rolle bei der Deletion von $CD8^+$ T Zellen während der HCV Infektion spielt, da die Aktivierung von T Zellen in der Leber von Mäusen und während der humanen HBV Infektion zu einer Hochregulierung von Bim führt. Im Gegensatz zu HBV war Bim in $CD8^+$ T Zellen in chronischen HCV Patienten *ex vivo* nicht anders reguliert als in Patienten mit ausgeheilter Infektion. Nach Antigen-spezifischer Aktivierung waren sowohl Bim als

auch der Aktivierungsmarker CD38 unabhängig vom Infektionsausgang hochreguliert. Zusammengenommen weisen unsere Daten darauf hin, dass während einer chronischen HCV Infektion die Dysregulation von Bim nicht zur Deletion von HCV-spezifischen $CD8^+$ T Zellen beiträgt.

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9 List of abbreviations

aa	amino acids
Abs	antibodies
ADAR1	adenosine deaminase 1
APC	allophycocyanin
BD	Beckton Dickinson
Bcl-2	B cell lymphoma-2
BFA	Brefeldin A
Bim	Bcl-2-interacting mediatior of death
CD	cluster of differentiation
cDNA	complemetary DNA
CI	confidence interval
CLDN1	claudin-1
cLDs	cytoplasmic lipid droplets
CTLA-4	cytotoxic T lymphocyte antigen 4
Cy	cyanin
DGAT-1	diacylglycerol acyltransferase-1
DAAs	direct-acting antivirals
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide Triphosphate
DPBS	Dulbecco's Phosphate-Buffered Saline
ds	double-stranded
eBio	eBioscience
EGFR	epidermal growth factor receptor
eIF	eukaryotic translation initiation factor
EMCDDA	European Monitoring Center for Drugs and DrugAddiction
EphA2	ephrin recpetor type A2
ER	endoplasmatic reticulum
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GAG	glycoasaminoglycans
GT	genotype
HBV	Hepatitis B virus
HCV	hepatitis C virus
HCC	hepatocellular carcinoma
HDL	high-density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	human leukocyte antigen
HVR	hypervariable region
ICS	intracellular cytokine staining
IDU	injection drug use
IFN	interferon
IFNAR	type I IFN receptor

IL	interleukin
IRES	internal ribosomal entry site
IRF-3	IFN regulatory factor-3
ISG	IFN-stimulated gene
IU	international units
JAK	Janus kinase
KIR	killer cell immunoglobulin-like receptor
KLRG1	killer-cell-lectin-like receptor G1
LDL	low-density lipoprotein
LSECs	liver sinusoidal endothelial cells
MAVS	mitochondrial antiviral signalling protein
MHC	major histocompatibility complex
MIP-1β	macrophage inflammatory protein
mRNA	messenger RNA
n.a.	not applicable
nAbs	neutralizing antibodies
n.d.	not done
NF-κB	nuclear factor-κB
NK	natural killer
NPC1L1	Niemann-Pick C1–like 1
OAS1	2'–5' oligoadenylate synthetases
OCLN	occludin
OMT	opioid maintenance treatment
OR	odds ratio
ORF	open reading frame
PAMPs	pathogen-assocaited molecular patterns
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PD-1	programmed death 1
pDCs	plasmacytoid dendritic cells
PD-L1	programmed death-ligand 1
PE	phycoerythrin
PerCP	peridinin chlorophyll
PKR	protein kinase R
PRRs	pattern recognition receptors
PWID	people who inject drugs
RdRp	RNA-dependent RNA polymerase
RIG-I	retinoic acid-inducible gene-I
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	reverse transcriptase
SD	standard deviation
SN	seronegative
SNPs	single nucleotide polymorphisms
SOCS3	suppressor of cytokine signalling 3
SR-B1	scavenger receptor class B type I
SSP	sequence-specific primers

STAT	signal transducer and activator of transcription
SVR	sustained virological response
TCR	T cell receptor
TGF	transforming growth factor
TIM-3	T cell immunoglobulin domain and mucindomain 3
TIR	Toll/IL-1 receptor
TLR	toll-like receptor
TNF	tumour necrosis factor
TRAF3	TNF-receptor associated factor 3
Treg	regulatory T cell
TRIF	TIR-domain-containing adaptor-inducing IFN-β
UTR	untranslated region
WHO	World Health Organization

10 List of figures

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12 Publications

Ziegler, S., Skibbe, K., Walker, A., Ke, X., Heinemann, F. M., Heinold, A., Mok, J. Y., van Esch, W. J. E., Yang, D., Wölfl, M. and Timm, J. Impact of sequence variation in a dominant HLA-A*02-restricted epitope in HCV on priming and cross-reactivity of CD8+ T cells.
Manuscript submitted to Journal of Virology

Walker, A.*, Skibbe, K.*, Kuntzen, T., Megger, D. A., Groten, S., Sitek, B., Lauer, G. M., Kim, A. Y., Allen, T. M. and Timm, J. Distinct escape pathway by HCV genotype 1a from a dominant CD8+ T cell response by selection of altered epitope processing.
Manuscript in revision in Journal of Virlology
*equal contribution

Ruhl, M., Chhatwal, P., Strathmann, H., Kuntzen, T., Bankwitz, D., Skibbe, K., Walker, A., Heinemann, F. M., Horn, P. A., Allen, T. M., Hoffmann, D., Pietschmann, T., Timm, J. Escape from a dominant HLA-B*15-restricted CD8+ T cell response against hepatitis C virus requires compensatory mutations outside the epitope. *J. Virol.* **86,** 991–1000 (2012)

13 Presentations

06/2014 "11th HepNet Symposium" in Hannover, Germany
poster presentation

04/2014 "49th Annual Meeting of the European Society for the Study of the Liver" in London, England
poster presentation

03/2014 "24th Annual Meeting of the Society for Virology" in Alpbach, Austria
poster presentation

03/2013 "23rd Annual Meeting of the Society for Virology" in Kiel, Germany
poster presentation

10/2012 "19th International Symposium on Hepatitis C Virus and Related Viruses" in Venice, Italy
poster presentation

03/2012 "22nd Annual Meeting of the Society for Virology" in Essen, Germany
poster presentation

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15 Curriculum vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

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16 Declarations

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, f der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Influence of the hepatitis C genotype on the antiviral immune response“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Kathrin Skibbe befürworte.

Essen, den _____ _____

Prof. Dr. Jörg Timm

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, c und e der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

Essen, den _____ _____

Kathrin Skibbe

Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, d und f der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe, dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist, und dass ich die Dissertation nur in diesem Verfahren einreiche.

Essen, den _____ _____

Kathrin Skibbe